CELLULAR IMMUNE RESPONSES TO HISTOCOMPATIBILITY ANTIGENS IN THE RAT

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Abstract

This project was concerned with certain immune responses mediated by T lymphocytes against major histocompatibility antigens of the rat. A cytotoxic population of lymph node cells was raised by grafting allogeneic skin and removing the draining lymph nodes eight days later. These cells were incubated on a monolayer of allogeneic lymphocytes which had been firmly attached to a polystyrene Petri dish pre-treated with poly-l-lysine. The immune cells were physically separated into a major non-adherent fraction and a minor adherent fraction. Most of the latter could be removed for testing with an EDTA solution. In confirmation of other work it was found that the non-adherent fraction was selectively depleted of cytotoxic activity; moreover, the adherent fraction was selectively enriched for this activity. The antigenic specificity of the separation was confirmed by using monolayers of lymphocytes from different inbred strains. Unexpectedly, the small number of lymph node cells adherent to a 'third-party' alloantigen were significantly less cytotoxic than the starting population.

The main point of the work was to measure as precisely as possible the graft-versus-host (GvH) activity of the adherent and non-adherent cell populations in order to determine whether GvH activity was partitioned in the same way as was cytotoxic activity. Simultaneous measurements of the two T cell activities produced a decisive answer: within the limits of detection there was no partition of GvH activity, since the adherent cells, the non-adherent cells and the starting population were all precisely equal in this respect.

The simplest explanation for these results requires three assumptions: (a) In an immune population, GvH reactive cells and cytotoxic cells belong to separate subsets; (b) Although GvH reactive cells display surface receptors for alloantigens, these are not able to bring about partition in the *in vitro* system described here; (c) The cytotoxic and GvH reactive

cells do not inteact in the functional tests. These assumptions were generally supported, but not definitely proved, by a number of supplementary experiments including measurement of GvH activity against 'third-party' alloantigens. The GvH activity of nonimmune lymph node cells was also identical in the adherent and non-adherent subpopulations.

A radioisotopic labelling method, which has been used to estimate the proportion of T cells responding to a given major alloantigen complex in a systemic GvH reaction, was modified in order to estimate the proportion of immune cells which adhered to allogeneic monolayers as a consequence of antigen recognition. A population of cells which had been immunised against one major alloantigen was labelled with either ^{3}H- or ^{14}C-uridine, and a population immune against a third-party alloantigen was labelled with the alternative isotope. The ratio of ^{3}H to ^{14}C in the adherent fraction, particularly that subfraction which was eluted with EDTA, always reflected the increased binding of the homologous immune population. On average, approximately 3% of the uridine-labelled cells were specifically adherent. This is of the same order as estimates of the proportion of cytotoxic cells in immune populations. However, when similar experiments were performed with immune populations labelled <u>in vitro</u> with ^{3}H- or ^{14}C-thymidine, it was found that, on average, 20% of the DNA synthesising population was specifically adherent. This suggests that most of the specifically adherent cells belong to the actively proliferating population, which others have shown to include most of the cytotoxic activity at this stage of the response. Nevertheless, the precise relationship between specific adherence <u>in vitro</u> and cytotoxicity has not been determined.

Several explanations are considered for the <u>in vitro</u> observation that cytotoxic T cells selectively adhere to the appropriate antigens

while GvH reactive cells do not. The individual receptors for antigen may have higher affinity or, alternatively, the cytotoxic cell may have higher avidity because it has a greater density of receptors. A third explanation is that the cytotoxic cell reacts to the engagement of its receptors by a non-specific increase in its adhesiveness. The only possibility which these experiments rule out is that the cytotoxic cell is more adherent before encountering antigen and that antigen-specific binding adds to this to make the total adherence to allogeneic cells greater than is the case with GvH reactive cells.

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CHAPTER ONE

INTRODUCTION

The cellular immune response which follows the transplantation of a solid tissue graft or cells from one individual of a species to another individual of the same species can be considered to consist of three phases; (1) the afferent limb of the response, which involves encounter with and recognition of antigen by immunocompetent lymphocytes; (2) the central phase, which entails the clonal proliferation and differentiation of the antigen-sensitive cells; and (3) the efferent limb or destructive phase of the response, in which the effector cells produced as a consequence of antigenic stimulation bring about the destruction of the foreign tissue or graft.

In recent years, two different models have provided detailed information on the sequence of events which follows the encounter of immunocompetent lymphocytes with the antigens determined by the major histocompatibility complex (major alloantigens) and on the functional activities of the lymphocytes produced as a result of antigenic stimulation.

One such model is the systemic graft-versus-host (GvH) reaction which follows the injection of lymphoid cells from one inbred strain of mice or rats into an F_1 hybrid between the donor strain and another inbred strain. A series of experiments performed by Gowans and co-workers (Gowans 1962, Ford et al. 1966) showed that, when radioactively labelled parental strain thoracic duct lymphocytes were injected intravenously into F_1 hybrid rats, the donor cells initially followed the normal migration pattern of lymphocytes injected into syngeneic recipients (Gowans and Knight 1964, Parrot et al. 1966) and localised in the thymus-dependent areas of the spleen and lymph nodes. A large minority of these cells subsequently transformed into large pyroninophilic blast cells which divided repeatedly, and evidence

was presented that this cell division resulted in the production of a new population of small lymphocytes. These findings were confirmed and extended in more recent studies in the mouse (Sprent and Miller 1972a) and in the rat (Atkins and Ford 1975) in which the F_1 hybrid recipients were irradiated prior to injection of H2- or AgB-incompatible thoracic duct lymphocytes or thymus cells, in order to permit more detailed observations regarding the fate of the injected cells. Following localisation and blast transformation, the activated lymphocytes proliferated within the spleen for several cell cycles; during the first 36 hours after injection in both species the thoracic duct lymph contained few donor cells and it was shown in the rat that the donor cells which were not retained within the lymphoid tissues, but which recirculated into the lymph during this time, were specifically unresponsive in GvH assays against the host alloantigens (Ford and Atkins 1971).

Two to four days after injection, the progeny of the activated cells left the lymphoid organs and some at least entered the recirculating pool. In both species approximately half of the donor cells which appeared in the thoracic duct were large lymphocytes and it was shown in the mouse that nearly 100% of these cells, large and small, had passed through at least one phase of DNA synthesis after injection into the recipient and had the characteristic features of T cells, whether derived from activation of thymus cells or thoracic duct lymphocytes (Sprent and Miller 1972a). Moreover, these cells were cytotoxic <u>in vitro</u> for tumour target cells which expressed the host alloantigens, were able to suppress the growth of the same tumour <u>in vivo</u>, and brought about accelerated graft rejection after transfer to previously skin grafted T cell-deprived mice (Sprent and Miller 1972). These cells did not display any of these immune activities towards

alloantigens other than those carried by the F_1 hybrid.

An <u>in vitro</u> model which has also provided detailed information on the response of lymphocytes to major histocompatibility antigens is the one-way mixed lymphocyte culture (MLC). When lymphocytes from non-immunised parental strain mice and rats are cultured with F_1 hybrid lymphocytes, the parental strain lymphocytes respond to the alloantigens of the F_1 hybrid by a vigorous cell proliferation which can be measured by the amount of ^{3}H-thymidine incorporated into the dividing cells (Dutton 1965, Wilson 1967). An extensive series of experiments in the rat was responsible for establishing that there was a requirement for AgB-incompatibility between the responding and stimulator cells (Wilson 1967, Sorensen 1972) and that parental lymphocytes from specifically tolerant or neonatally-thymectomised rats did not proliferate in response to F_1 alloantigens (Wilson et al. 1967). Two independent groups subsequently made the important observation in man (Solliday and Bach 1970) and in the mouse (Häyry and Defendi 1970) that stimulation of lymphocytes in one-way MLC resulted in the production of cytotoxic lymphocytes and, by exposing the cultures to agents which selectively damage dividing cells, it was shown that there was a requirement for cell proliferation in the production of cytotoxic lymphocytes (Bach et al. 1972, Häyry et al. 1972). It was subsequently found in the mouse that nearly pure populations of T cells, such as thymus cells (Wagner 1971, Lohmann-Mathes and Fischer 1972) or spleen cells depleted of B cells by different procedures (Häyry et al. 1972, Wagner et al. 1972), responded in one-way MLC and generated cytotoxic cells. Moreover, the cytotoxic cells produced were susceptible to anti-Θ serum and complement, confirming that they were T cells (Tyan and Ness 1972, Wagner et al. 1972). In contrast, spleen cells

from thymectomised, bone marrow-reconstituted mice (Feldman et al. 1972) and cell populations depleted of T cells by treatment with anti-θ serum and complement (Wagner et al. 1972) did not generate cytotoxic cells in vitro.

Each of the responses described have several important features in common: first, both are initiated predominantly if not wholly by the thymus-derived lymphocyte population; secondly, both are stimulated by the antigens determined within the major histocompatibility complex; and thirdly, the progeny of the lymphocytes which proliferate in response to antigenic stimulation includes specifically cytotoxic T cells.

This thesis is concerned with the relationship between the virgin T cells which proliferate in response to major alloantigens and the cytotoxic cells produced as a consequence of stimulation with antigen. In this context, the non-immune T cells which cause or provoke GvH or MLC reactions have been called initiator lymphocytes (Howard 1973), with the implication that initiator and effector cells mediate respectively the afferent and efferent phases of the alloantigen response. Although the responses of initiator and cytotoxic lymphocytes to antigen are clearly different, viz. proliferation and target cell destruction respectively, it can be demonstrated that each is specific for antigens determined within the major histocompatibility complex and the simplest hypothesis is that the receptors for antigen on the two cell types are the same.

Initiator and cytotoxic lymphocytes can be further distinguished on the basis of size, their probable life-span and possibly in their migratory properties. A number of studies in the mouse and in the rat clearly established that the small lymphocytes of the recirculating pool are responsible for the initiation of GvH reactions. The majority

of small lymphocytes in the blood and lymph are long-lived, nondividing cells; the population only gradually accumulates labelled cells during prolonged infusion of ^{3}H-thymidine *in vivo* (Everett et al. 1964), and their life-span, i.e. the time from one mitosis to the next mitosis or cell death, has been estimated in terms of months or years depending on the species (Robinson et al. 1965, Buckton et al. 1967). Gowans (1962) showed that the severity of the GvH reaction in F_1 hybrid rats injected with parental thoracic duct lymphocyte populations which contained different numbers of large and small lymphocytes was a function of the number of small lymphocytes, and that removal of the large lymphocytes prior to injection did not result in a reduced GvH activity. Similarly, Hildemann et al. (1962) showed that pure populations of peripheral blood small lymphocytes were effective in inducing GvH reactions in neonatal F_1 hybrid mice. The finding that thoracic duct drainage in the mouse reduced the GvH activity of spleen cells taken from these animals (Cantor et al. 1970) also suggested that the recirculating lymphocytes made a critical contribution to the GvH activity of the lymphoid tissues.

In contrast, cytotoxic lymphocytes are heterogeneous in size, may be short-lived cells and probably recirculate poorly from blood to lymph. Physical characterisation of cytotoxic lymphocytes in the spleens of mice undergoing tumour allograft rejection revealed significant differences in size and density between cytotoxic cells obtained early or late after immunisation; the former were predominantly large and of low density corresponding to blast cells, whereas the latter were mainly small and dense (Pelet et al. 1971, Shortman et al. 1972). Similar results were also obtained in studies of cytotoxic lymphocytes generated *in vitro*; velocity sedimentation analysis of cytotoxic cells

produced in MLC showed that at the peak of the response cytotoxic activity was associated with medium to large lymphocytes, whereas later in the response the cytotoxic cells possessed the sedimentation characteristics of small lymphocytes (MacDonald et al. 1973).

The fairly rapid decline in cytotoxic activity in the lymphoid organs following immunisation with alloantigens suggests that cytotoxic cells are relatively short-lived. No cytotoxic activity is detectable 2-3 weeks after skin graft rejection in mice and rats (Canty and Wunderlich 1971, Peter and Feldman 1972), and two to three months after injection of living allogeneic tumour cells in mice (Brunner et al. 1970). However, recent studies have suggested that loss of detectable cytotoxic activity may not necessarily be a consequence of the death of the cytotoxic cells, but may instead be associated with reversion of these cells to 'memory' lymphocytes which are no longer cytotoxic but which regain their cytotoxic activity following restimulation with antigen (vide infra).

There are a number of pieces of evidence to suggest that cytotoxic lymphocytes display an altered migration pattern <u>in vivo</u> in comparison to the small lymphocytes of the recirculating pool. Sprent and Miller (1972b) showed that the cytotoxic cells which appear in the thoracic duct of irradiated recipients of semi-allogeneic thymus cells did not, for the most part, return to the thoracic duct if labelled and reinjected into syngeneic animals but migrated instead into the small intestine. More recently, Häyry and Andersson (1975) showed that when mouse cytotoxic cells generated in MLC were transferred to irradiated syngeneic recipients, cytotoxic activity was detectable in the spleen but not in lymph nodes, suggesting that these cells were not fully capable of recirculating. In both cases the cytotoxic cell populations

comprised mainly blast cells and the altered migration patterns described may reflect the general tendency of large newly-formed lymphocytes, whether T cells or B cells, to leave the circulation and to accumulate non-specifically in inflammatory exudates (McGregor et al. 1971, Koster et al. 1971) or in the lamina propria of the gut (Gowans and Knight 1964, Hall et al. 1972).

The simplest hypothesis concerning the relationship between initiator and cytotoxic lymphocytes is that the virgin lymphocytes which proliferate in response to each major histocompatibility complex include the precursors of cytotoxic cells, and that cytotoxic lymphocytes are produced by a process of clonal proliferation and differentiation of this subpopulation of lymphocytes. However, there is now considerable evidence that both GvH and cell-mediated cytotoxic responses may involve the participation of more than one T cell subset.

Evidence that two subpopulations of T cells are involved in GvH reactions was obtained by Cantor and Asofsky (1970, 1972), who found synergy between thymus cells and either blood lymphocytes or lymph node cells in producing splenomegaly in neonatal F_1 hybrid mice. Both the cell populations were required to be responsive to the alloantigens against which the response was directed, were depleted by neonatal thymectomy and were θ-positive (Cantor 1972); and it was postulated that two distinct T cell types were involved, one of which (T_1) contained the effector cell precursors, the other (T_2) acting as an amplifier or helper cell. In subsequent experiments, Tigelaar and Asofsky (1973) demonstrated synergy in GvH reactions between apparently distinct T cell populations present in the same lymphoid organ viz. the spleen. Following injection of spleen cells from normal mice into syngeneic lethally-irradiated recipients, one

subpopulation accumulated in the lymph nodes whereas the other accumulated predominantly in the spleen. Both the 'lymph node seeking' and 'spleen seeking' cells were required to restore the GvH activity to that of whole spleen cell populations and to demonstrate the synergistic effect; cell populations recovered from either the spleen or lymph nodes when tested alone had markedly reduced GvH activity.

A number of different groups have also demonstrated synergy between murine thymus cells and lymph node cells in the production of cytotoxic lymphocytes in one-way MLC (Wagner 1973, Tigelaar and Feldman 1973, Cohen and Howe 1973). In each case limited numbers of either thymus cells or lymph node cells were unable to generate substantial cytotoxic activity in response to allogeneic spleen cells in vitro, whereas mixtures of the two cell populations generated high levels of cytotoxic activity. In addition, the results of one such study indicated that peripheral T cells provided the major source of cytotoxic cell precursors while the thymus cells acted mainly as amplifier cells in the MLC (Wagner 1973).

More recently, alloantigen reactive T cell subpopulations with a similar functional relationship have been distinguished in the mouse according to their expression of antigens belonging to the Ly-system (Cantor and Boyse 1975). Peripheral T cells can be separated into three populations on this basis viz. Ly-1,2,3 Ly-1 and Ly-2,3. By selectively depleting splenic T cells of the different Ly subpopulations by treatment with specific antisera and complement prior to stimulation with alloantigens in vitro, it was demonstrated that, although both Ly-1 and Ly-2,3 cells responded to alloantigens by proliferation in MLC, cytotoxic cells and their precursors belonged exclusively to the Ly-2,3 subclass. Although there was no evidence of an absolute requirement for both cell types for the production of cytotoxic lymphocytes, the addition

of Ly-1 cells to limited numbers of Ly-2,3 cells was found to augment the cytotoxic response.

Evidence for a T-T cell interaction in the in vitro generation of cytotoxic lymphocytes has also been obtained from analysis of the antigenic specificities within the major histocompatibility complex (MHC) which provoke cell proliferation in MLC and against which cytotoxic activity is directed. In both man (Bach et al. 1972a) and in the mouse (Yunis and Amos 1971), cell proliferation in MLC can be induced by the antigenic products of a genetic region within the MHC, the LD locus, which is closely linked to but distinct from the locus controlling the serologically defined (SD) major histocompatibility antigens. Cytotoxic cells are not, however, generated in vitro when the responder and stimulator cell populations differ only in LD antigens. By measuring cell proliferation and cytotoxic cell production in a variety of responder and stimulator cell combinations, using human families (Trinchieri et al. 1973) and intra-H2 recombinant mouse strains (Alter et al. 1973, Abbasi and Festenstein 1973), it was shown that there was a requirement for both LD and SD differences for the generation of cytotoxic lymphocytes. The cytotoxic lymphocytes produced, however, had a specificity directed exclusively against SD antigens and, furthermore, could be induced by a mixture of stimulator cells, one differing from the responder cells by SD determinants alone, the other differing by LD determinants alone (Eijsvoogel et al. 1973a). Support for the hypothesis that the cells which responded to LD and SD antigens were distinct was obtained from studies which showed that cytotoxic activity could be selectively removed by adsorption of lymphocytes on SD-different allogeneic cell monolayers, without affecting the capacity of these cells to proliferate in MLC (Bach et al. 1973).

It is of considerable importance to establish whether cytotoxic lymphocytes are terminally differentiated cells with restricted immunological function, or whether cytotoxic activity is a property of activated T cells at a particular stage of maturation or differentiation. Although it was demonstrated many years ago that skin allografts were rejected more rapidly by specifically immunised mice (Medawar 1946) and that allograft immunity could be adoptively transferred to non-immune animals by specifically sensitised lymphocytes (Billingham et al. 1954, Mitchison 1954), the cellular basis of memory to major histocompatibility antigens is not yet fully understood. Studies which compared the activity of normal and alloimmune lymphoid cell populations in graft-versus-host assays in vivo (Simonsen 1962, Ford and Simonsen 1971), or by a proliferative assay in vitro (Wilson and Nowell 1971), failed to detect significant differences between the two cell populations, suggesting that the numbers of reactive cells was not substantially increased by immunisation. In contrast, anamnestic cytotoxic responses, characterised by an accelerated appearance and higher levels of cytotoxic activity, have been demonstrated in studies in which the ability of normal and alloimmune spleen cells to respond to the relevant alloantigens both in vivo (Canty and Wunderlich 1971) and in vitro (Cerottini et al. 1974) was quantitatively compared. Taken together, these findings imply that T cell memory involves qualitative rather than simply quantitative differences between primed and non-primed cell populations and suggest that cytotoxic lymphocytes themselves may be implicated in the secondary response to major alloantigens.

Since there is no method for identifying individual cytotoxic lymphocytes, the exact relationship between memory and effector cells cannot be directly assessed. However, there is indirect evidence that

cytotoxic lymphocytes have only a limited capacity to respond to antigen by proliferation. Sprent and Miller (1972) showed that highly cytotoxic cell populations, obtained from the thoracic duct of irradiated recipients of semiallogeneic thymus cells, were unable to cause splenomegaly in neonatal F_1 hybrids carrying the immunising alloantigens. Subsequent experiments showed that although these cells incorporated ^{3}H-thymidine in response to specific antigen in vitro, the majority of cells which did so did not subsequently enter mitosis (Cheers et al. 1973). Other groups have also failed to induce proliferative responses, whether tested by GvH or MLC, in cell populations harvested from one-way MLC at the peak of the cytotoxic response (Rouse and Wagner 1972, MacDonald et al. 1974).

However, with refinements in tissue culture techniques it has recently become possible to maintain cells in mixed lymphocyte culture for considerable periods of time and it was found that re-exposure of cells in long term MLC to the original stimulating antigens, at a time when cytotoxic activity was no longer detectable, resulted in significant cell proliferation and rapid regeneration of cytotoxic lymphocytes (MacDonald et al. 1974). Subsequent studies using velocity sedimentation to separate cells in MLC on the basis of size showed that the cells which responded to a secondary antigenic stimulus by the formation of cytotoxic cells were small lymphocytes, and that these cells were derived from the blast cells present in primary MLC at the peak of the response (MacDonald et al. 1974a, Andersson and Häyry 1974). Since cytotoxic activity is associated almost exclusively with the blast cell population at this time, these results raised the possibility that the cytotoxic cell progenitors in long term MLC were the progeny of the cytotoxic lymphocytes generated during the primary response. If so, then cytotoxic cells cannot be considered to be terminally differentiated 'end-cells'.

However, without a means of identifying or isolating individual cytotoxic lymphocytes, the possibility that the cells which responded to secondary challenge were derived from an independent, but physically similar, activated cell population unrelated to the primary cytotoxic cells could not be excluded.

One possible means of circumventing this problem is to use immuno-adsorption on allogeneic cell monolayers as a method for obtaining a potentially pure population of cytotoxic lymphocytes. Specific depletion of cytotoxic cells has been accomplished by adsorption of immune cell populations on allogeneic monolayers of several cell types (Brondz 1968, Golstein et al. 1971) and the results of two such studies suggested that cytotoxic cells adhered to the monolayers under conditions in which other functional populations of alloantigen-reactive T cells did not (Bach et al. 1973, Mage and McHugh 1973).

The principal object of the work presented here was to partition alloimmune lymph node cells on an allogeneic cell monolayer into adherent and non-adherent cell populations which could then be simultaneously tested for their capacity to respond to specific antigen in both cytotoxic and proliferative assays. A number of supplementary experiments, particularly specificity controls, were necessary for interpretation of the results obtained. The experiments presented in Chapter 3 describe the development of a quantitative assay for cytotoxic T cells in the rat. In the following chapter, this assay was used to obtain a quantitative assessment of the depletion of and enrichment in cytotoxic activity of lymphoid cell populations separated by adsorption on allogeneic lymphocyte monolayers. The effect of such an enrichment and depletion in cytotoxic activity on the capacity of the same cell populations to initiate a GvH reaction against the relevant alloantigens is described in Chapter 5.

CHAPTER TWO

MATERIALS AND METHODS

Animals

Five highly inbred rat strains were used viz. AO (Ag-B2), WF (Ag-B2), DA (Ag-B4), HO (PVG/$_c$, Ag-B5), (Festing and Staats 1973), and the congenic HO.B2 (Ag-B2) which has the genetic background of the HO strain, differing only at the AgB locus (Chisholm et al.). F_1 hybrids between pairs of these strains were also used. The animals were bred and maintained under conventional conditions in the Animal Unit, Manchester University Medical School and previously at the Department of Pathology, Edinburgh University Medical School.

Skin grafting

Full thickness, 2 cm. square, orthotopic skin grafts were sutured with 5/0 silk (W500, Ethicon, Scotland) into beds prepared on the lateral thoracic wall. The grafts were covered with a sterile dressing (Sofra Tulle, Roussel, England) and secured by wrapping gauze bandage followed by adhesive bandage round the animal, from just below the axilla to the lower end of the rib cage. The dressings were left in place until the animals were killed and the lymphoid tissues removed (usually 6-8 days).

Tissue culture

(a) WF/G$_1$ lymphoma cell line. The WF/G$_1$ lymphoma is a cell line established from the Gross virus-induced C58NTD lymphoma of Wistar Furth rats, and was kindly supplied by Dr. G. Shellam, Department of Zoology, University College, London. The cells were grown in suspension culture in RPMI 1640 Hepes (20mM)-buffered medium, supplemented with glutamine (20mM), penicillin/streptomycin (100 units/ml., 25 mg./ml.) and 10% foetal calf serum (FCS). The cells were maintained in the exponential phase of growth (1-8x10^5 cells/ml.) by serial passage three times weekly.

(b) Primary rat fibroblasts. Primary fibroblast cultures were

obtained from foetal lung (14-21 days gestation) and were maintained in RPMI 1640 supplemented as above, with the exception that the FCS concentration was 15%. The cells were grown in flat-sided glass bottles or in plastic tissue culture flasks (Falcon Plastics 3013, Gibco-Biocult, Scotland) and passaged once weekly. The cells were used in all cases after at least two passes, by which time a uniform cell population had been established.

Lymphoid cell suspensions

(a) Lymph nodes. Lymph nodes were excised, cleaned of adhering fat and teased with scalpel blades in RPMI 1640 + 10% FCS. The cells were filtered through a fine stainless steel mesh and the pieces of tissue gently pressed through the mesh to free any cells remaining. The cell suspension was left at 4°C to allow small debris to settle and the single cell suspension was decanted. The cells were washed twice in RPMI 1640 + 10% FCS (500g,10mins).

(b) Spleen cells. Spleen cell suspensions were prepared as described for lymph nodes.

(c) Thoracic-duct lymphocytes (TDL). The surgical procedure for the cannulation of the rat thoracic duct and the conditions under which the rats were maintained after surgery was based on Gowan's modification of Bollman's method as fully described by Ford and Hunt (1973). The thoracic duct lymph obtained was filtered through gauze to remove any blood or fibrin clots, centrifuged (500g,10mins), and the TDL resuspended in RPMI 1640.

Labelling of lymphocytes in vitro.

The method used for labelling lymphocytes in vitro was basically the same for the different isotopes used. In all cases the cells to be labelled were resuspended at the desired concentration in RPMI 1640 Hepes-buffered medium + 10% FCS, in either 25 ml. conical flasks or

10 ml. conical centrifuge tubes. The cells were incubated with the isotope for one hour at 37°C in a water-bath and the flasks or tubes gently shaken by hand at 20 min. intervals. After labelling, the cells were washed three times (500g,10mins.) in RPMI 1640 + 10% FCS. Details of the labelling regime for the different isotopes and cells are given in the accompanying table:

Isotope (Radiochemical Centre Amersham)	Cells	Isotope concentration
(6-^{3}H) Thymidine TRA.61 5000 mC/mM	Lymph node TDL $2-5 \times 10^{7}$/ml.	0.4 µCi/ml
(2-^{14}C) Thymidine CFA.219 > 50 mC/mM	Lymph node TDL $2-5 \times 10^{7}$ml.	0.6 µCi/ml
(5-^{3}H) Uridine TRK.178 2000-5000 mC/mM	Lymph node TDL $2-5 \times 10^{7}$ml.	7 µCi/ml
(U-^{14}C) Uridine CFB.51 > 405 mC/mM	Lymph node TDL $2-5 \times 10^{7}$/ml.	1.0 µCi/ml

Labelling of target cells for the cytotoxic assay.

(a) ^{125}I-iododeoxyuridine (^{125}IUdR).

The WF/G_1 lymphoma cells were labelled in culture (exponential phase of growth 1-2 x 10^5 cells/ml) for 24 hours with 0.04 µCi/ml ^{125}IUdR (IM 352, 1-6 mCi/mg, Radiochemical Centre, Amersham). The cultures were pooled and the cells were washed four times (300g,10mins.,4°C) in RPMI 1640 + 10% FCS. The cells were kept at 4°C after labelling; this was found to substantially reduce the spontaneous release of the isotope from the cells during the period of the cytotoxic assay. More than 95% of the cells were labelled under these conditions as assessed by autoradiography.

(b) ^{51}Cr-Sodium Chromate ($Na_2\ ^{51}CrO_4$)

(i) Fibroblasts.

Primary fibroblast cultures were maintained as described. The cells were recovered from the culture flasks by trypsinisation, were washed twice in RPMI 1640 + 10% FCS (300g.10mins.), resuspended in the same medium (1.0 x 10^6 cells/ml) and labelled in suspension at 37°C for one hour with 100 µCi/ml ^{51}Cr-sodium chromate (CJS IP. 50-400 mCi/mg Cr, Radiochemical Centre, Amersham). The cells were washed three times after labelling with RPMI 1640 + 10% FCS and kept at 4°C until required.

(ii) WF/G_1 lymphoma.

Suspension cultures were centrifuged (300g.10mins.) and the cells washed twice in RPMI 1640 + 10% FCS. The cells were resuspended in the same medium at 2.0 x 10^7 cells/ml, and labelled with 50 µCi/ml ^{51}Cr-sodium chromate at 37°C for one hour. The cells were washed four times after labelling in RPMI 1640 + 10% FCS (300g.10mins.4°C).

Preparation of cell suspensions for scintillation counting.

Whereas the measurement of γ emission from radioactively labelled lymphocytes requires no sample preparation, the measurement of weak β emission from ^3H and ^{14}C in lymphocytes requires a laborious and time-consuming preparation.

(1) Lymphocyte pellets were resuspended in not more than 0.5 ml. saline, transferred to screw-capped plastic counting vials and 0.5 ml. 2M NaOH added. The mixture was incubated in a water-bath at 80°C for 15 mins.

(2) The suspension was cooled and bleached by adding 0.3 ml. of 100 volumes per cent H_2O_2 (BDH Ltd.) and leaving the samples overnight with loosened caps to allow the escape of O_2 evolved.

(3) The samples were then acidified by adding 0.3 ml. conc.HCl,

and 15 ml. of cold (4°C) scintillant were added.

(4) The vials were then shaken vigorously, wiped clean and left overnight in the scintillation counter before counting was started.

Scintillant.

667 ml.	Toluene	BDH Ltd.
333 ml.	Triton-X-100	
5.4 g	Butyl-PBD	CIBA Ltd.
0.3 g	PBBO	

The Cytotoxic Assay.

The method described here is the one which was found to be the most satisfactory, by several criteria (see Ch.3.1), for the measurement of cell-mediated cytotoxicity.

The assay was performed in flat-bottomed microtitre plates (Nunclon N1480). All cell suspensions were prepared in RPMI 1640 Hepes-buffered (20 mM) medium, supplemented with glutamine (20 mM), penicillin/streptomycin (100 units/ml) and 15% heat-inactivated FCS (56°C, 45 mins.). Serial doubling dilutions of immune lymphocyte suspensions were prepared in the assay medium and the target cells used were WF/G_1 lymphoma cells pre-labelled with ^{125}IUdR as described.

The immune cells were added in 0.1 ml. volumes to the wells of the microtitre plates. Each dilution was tested in triplicate. 0.1 ml. of the target cell suspension (2.5×10^5 cells/ml) was then added to all the wells. The plates were centrifuged (20g, 5mins.), covered with a loose-fitting lid and incubated at 37°C for 18 hours. The plates were then centrifuged (300g, 15 mins.) and 0.1 ml. of the supernatant medium from each well was removed for counting in a gamma counter (L.K.B.).

Controls in all assays consisted of:-

(1) Target cells incubated with non-immune lymphocytes at a concentration equal to the highest immune cell number tested (Background Release).

(2) Target cells alone in 0.2 mls. medium (Spontaneous Release).

(3) Target cells in 0.2 mls. detergent (0.6% Sodium Dodecyl Sulphate) (Detergent Maximum Release).

Cytotoxicity was calculated as

$$\text{Per cent specific } {}^{125}\text{I-release} = 100 \times \frac{(I - N)}{(M - N)}$$

where I = cpm immune cell supernatant

N = cpm non-immune cell supernatant

M = cpm detergent supernatant

The background release was 10-15% of maximum release after 18 hours incubation and the spontaneous release was marginally higher at 15-20%. The maximum release was 85-95% of the total incorporated isotope.

The Graft-vs-Host Assay.

Lymphocytes were tested for Graft-vs-Host activity using the popliteal lymph node weight assay described by Ford Burr and Simonsen (1970). Parental strain lymphocytes in 0.1 ml. Dulbeco's phosphate-buffered saline (DAB, Oxoid Ltd.) were injected subcutaneously into the hind foot-pad of young (6-8 weeks old) F_1 hybrid rats. Two doses (routinely 3.0 and 1.0 x 10^6 cells) of each cell population were tested. and four feet were injected with each cell population at each dose. Animals were matched for age and sex within each experimental group. Control animals were injected with 0.1 ml. DAB. Seven days after injection, the popliteal lymph nodes were excised, carefully cleaned and weighed.

A quantitative comparison of the GvH activity of different lymphoid cell populations was obtained by comparing the numbers of lymphocytes in a given (test) cell population required to produce the same lymph

node enlargement as a control population as described by Michie (1973). The results were expressed as potency ratios; a potency ratio of greater than 1.0 indicates that the test cell population is more powerful on a per cell basis than the control population.

Separation of Lymphocyte populations by Adsorption on Thoracic-duct Lymphocyte Monolayers.

(a) Monolayer preparation.

Lymphocyte monolayers were prepared on poly-l-lysine (PLL)-treated tissue culture plates as described by Stulting and Berke (1973).

2.5 mls. of a poly-l-lysine solution (Sigma P1886 MWt. 70,000, 50 ug/ml in phosphate-buffered saline (PBS), pH 7.3) were added to 60 x 15 mm. polystyrene petri-dishes (Falcon Plastics 3002) and the plates were incubated at room temperature for one hour. The unbound PLL was removed and the plates thoroughly rinsed by dipping repeatedly in a beaker of PBS followed by rinsing four times with PBS. The plates were covered with fresh PBS and kept at $4^{\circ}C$ until required.

Thoracic-duct lymphocytes (TDL) were washed once in RPMI 1640 without added serum and were resuspended in the same medium. 2.5 mls. of the suspension, containing not less than 30×10^6 cells/ml., were added to each plate and the plates incubated at room temperature for 45 mins. Excess TDL were removed by repeated rinsing with PBS and the confluent monolayers covered with RPMI 1640 + 10% FCS and kept at room temperature until required.

(b) Adsorption.

Lymphocyte suspensions were fractionated on TDL monolayers by the method of Kedar et al. (1974a).

2.5 mls. of the lymphocyte suspension (6×10^6 cells/ml. in RPMI 1640 + 10% FCS) were added to each monolayer and the plates incubated at $37^{\circ}C$ for 30 mins. The plates were then stacked and taped

and centrifuged at 130 g for 5 minutes at 24°C.

The plates were gently agitated by hand and the non-adherent cells removed by pipetting the supernatant medium over the monolayers, removing the medium and rinsing the monolayers once with 2.5 mls. PBS. The cells and the rinse were combined to give the non-adherent cell fraction. Non-adherent cell recoveries were approximately 90% of the cells added.

The adherent cells were recovered by adding 2.5 mls. EDTA (BDH Ltd., 5 mM in PBS + 10% FCS) to each plate and incubating the plates at 37°C for 15 minutes. The adherent cells were removed as before by gentle manual agitation and rinsing of the monolayers with PBS. Approximately 60-70% of the adherent cells were eluted under these conditions.

The non-adherent and adherent cell populations were washed once in RPMI 1640 + 10% FCS (500g, 10mins.).

Depletion and Enrichment of Immunoglobulin-positive Lymphocytes.

(1) Ig anti-Ig column fractionation.

Lymphocyte populations were depleted of Ig^+ cells by passage through Ig anti-Ig columns by a modification of the method of Wigzell (1970) described by Shand and Bell (1976).

Diakon beads (Imperial Chemical Industries, Welwyn Garden City, U.K.), washed in distilled water then in phosphate-buffered saline (PBS), were incubated with rat Ig (a 50% saturated ammonium sulphate precipitate of normal rat serum) at 45°C for 1 hour, and allowed to stand overnight at 4°C. The Ig-coated beads were washed five times with PBS in the cold (4°C), poured into chromatographic columns (Pharmacia Fine Chemicals, Uppsala, Sweden) and equilibrated with PBS at 4°C for one hour. The column was then saturated with a 1 in 2 or a 1 in 3 dilution of a hyperimmune rabbit anti-rat Ig serum at 4°C and allowed to stand for 1.5 hours. The column was washed extensively (1 hour, 5 mls./min.) with PBS and the lymphocyte suspension (10^7 cells/ml. in RPMI 1640) added.

The cells were eluted from the column at the rate of 2-3 ml./min. and washed twice in RPMI 1640 + 10% FCS. Cell recoveries were approximately 40% of the cells added.

(2) Adsorption on Antibody-coated sheep erythrocyte monolayers.

The method described is essentially that of Kedar et al. (1974b) with a modification of the method for the recovery of the adherent cells.

Poly-l-lysine coated petri-dishes were prepared as described previously. 2.5 mls. of a 1% (v/v) suspension of sheep red blood cells (SRBC) washed five times in PBS were added to each plate. After incubation at room temperature for 45 minutes, excess SRBC were removed by repeated rinsing of the plates with PBS, until confluent, firmly adherent monolayers were obtained.

2.5 mls. of a rabbit anti-SRBC serum (Wellcome VD15, a 50% saturated ammonium sulphate precipitate, 1 in 50 dilution in PBS) were added to the plates and the plates incubated at 37°C for 30 mins. The serum was then removed and the monolayers washed extensively with PBS.

2.5 mls. of the lymphocyte suspension (10^7 cells/ml. in Dulbeco's phosphate-buffered saline (DAB, Oxoid Ltd.) + 10% heat-inactivated FCS) were added to each plate and after a 20 min. incubation at room temperature the plates were centrifuged at 130 g for 5 mins. The plates were gently agitated by hand and the non-adherent cells removed by pipetting the supernatant medium over the monolayers and removing the medium. The monolayers were then rinsed twice with PBS (these rinses were discarded). The adherent cells were recovered by adding 2.5 mls. guinea-pig complement (Wellcome freeze-dried, a 1 in 80 dilution in PBS) to each plate and incubating the plates at room temperature until lysis of the SRBC was complete as judged by microscopic observation (10-20 mins.). The remaining lymphocytes were harvested with a rubber policeman and resuspended in a large volume of DAB + 10% FCS. Both the non-adherent and adherent cells were washed twice in RPMI 1640 + 10% FCS.

CHAPTER THREE

THE PRODUCTION OF CYTOTOXIC LYMPHOCYTES IN RESPONSE TO SKIN ALLOGRAFTS.

The production of specifically sensitised cytotoxic T-lymphocytes as a consequence of allogeneic immunisation has been described in a number of species including mice (Rosenau and Moon 1961, Brunner et al. 1968), rats (Wilson 1963, Peter and Feldman 1972) and man (Solliday and Bach 1970), and a variety of different in vitro assays of cell-mediated cytotoxicity have been described. The most commonly used are those which assess target cell damage by measuring the release of radioactive label. The successful measurement of cell-mediated cytotoxicity (CMC) by this technique requires a suitable combination of isotope and target cell. The target cell must express the antigens concerned on its surface and must be susceptible to lysis once it has been damaged in the cytotoxic reaction. The isotope used to label the cells should be firmly bound within the cells so that spontaneous release from the cells in vitro is low, and the isotope should be released from the cells in a form which is not reutilised. A number of different isotopes have been used to label target cell protein (Bickis et al. 1959), RNA (Hashimoto and Sudo 1971) or DNA (Vaino et al. 1964, Cohen et al. 1971). ^{51}Cr was used as a routine label in studies on RBC survival in haemolytic diseases (Sanderson 1964) and was adapted for the quantitation of antibody-induced lysis of cells in vitro (Wigzell 1965). It was subsequently found to be a good label for the measurement of cell-mediated cytotoxicity (Holm and Perlmann 1967, Brunner et al. 1968) and has since become the most widely used. Almost all cells will incorporate the isotope in vitro and a variety of ^{51}Cr-labelled target cells have been used, including macrophages (Harder et al. 1974), fibroblasts (Berke et al. 1969) and a number of tumour cells (Brunner et al. 1968, Canty and Wunderlich 1970, Oren et al. 1971). ^{51}Cr is covalently bound to proteins and other cell constituents; the chromate is reduced during binding and the isotope is not reutilised (Bunting et al. 1963, Holm and Perlmann 1967).

The use of ^{51}Cr is chiefly limited by the rate of spontaneous release of the isotope from cells in vitro. For reasons which are

poorly understood, spontaneous release rates vary considerably from one cell type to another. Different target cells also vary in their susceptibility to lysis by the same cytotoxic cell population (Brunner et al. 1970). It may be that some cells which are poor targets for CMC express insufficient amounts of membrane alloantigens, but it seems that, apart from this, there are substantial differences in the inherent susceptibility or resistance of different cell types to cell-mediated lysis. Certain tumour cells, notably those of lymphoid origin, are highly susceptible to lysis and the most successful isotope-release assays have used mastocytoma (Brunner et al. 1968) or lymphoma cells (Canty and Wunderlich 1970, Oren et al. 1971) in suspension as target cells. It is perhaps significant in this respect that, whereas normal lymphocytes are poor target cells, mitogen-stimulated lymphocytes have recently been found to be satisfactory (Lightbody et al. 1971, Nabholz et al. 1974).

The production of cytotoxic lymphocytes after skin allografting has been described in mice (Canty and Wunderlich 1971) and rats (Peter and Feldman 1972). Cytotoxic activity, as assessed by ^{51}Cr-release assays, was detected in the draining lymph nodes, spleen and peripheral blood, and peak activity coincided with the rejection of the allogeneic skin graft. Direct evidence for the participation of specifically sensitised T-cells in skin graft rejection was obtained by Sprent and Miller (1972), who showed accelerated skin graft rejection in neonatally thymectomised mice following injection of a pure population of T-cells immunised against the graft alloantigens. This population was also highly cytotoxic <u>in vitro</u> in a ^{51}Cr-release assay.

In this chapter, the development of a sensitive assay for cell-mediated cytotoxicity in the rat is described. Using this assay, the cytotoxic response in the draining lymph nodes of skin allografted rats

was characterised in terms of the kinetics and specificity of the response. Evidence is presented that the cytotoxic activity measured under these conditions is a function of thymus-derived lymphocytes, and the major contribution of the antigens determined by the major histocompatibility complex (AgB) to this response is established.

RESULTS

3.1 The measurement of cell-mediated cytotoxicity.

The experiments presented in this section were carried out in order to establish optimum conditions for the measurement of cell-mediated cytotoxicity in the rat. Two different target cells were used viz. fibroblasts and a lymphoma cell line and these cells were labelled *in vitro* with either ^{51}Cr or ^{125}Iododeoxyuridine (^{125}IUdR). In all experiments immune lymphoid cells were obtained from skin allografted rats and, unless otherwise stated, the donors and recipients of the grafts differed atthe major histocompatibility locus AgB.

(a) ^{51}Cr-labelled fibroblasts as target cells.

AO rats were immunised with DA skin grafts and lymphocytes from the spleen and the draining lymph nodes were tested for cytotoxicity against ^{51}Cr-labelled fibroblasts. The results obtained in five separate experiments are presented in TABLE 1. There was considerable variation in the per cent specific ^{51}Cr release measured in different experiments. Lymph node lymphocytes gave the highest response; 36% specific ^{51}Cr-release at a lymphocyte/target cell ratio of 100:1, but the response was generally low. ^{51}Cr-release from target cells incubated with non-immune lymphocytes (background release) was 40-50% after 18 hours and the spontaneous release from fibroblasts cultured alone was also 40-50%. Since it was possible that there might be a greater difference between ^{51}Cr-release in the presence of immune cells and

TABLE 3.1.

CYTOTOXICITY AGAINST ^{51}Cr-LABELLED FIBROBLASTS

EXPT.	DAYS AFTER GRAFTING [a]	PER CENT SPECIFIC ^{51}Cr-RELEASE [c] LYMPH NODE [b]	SPLEEN
1	7	14.4 ± 2.5 (44.0)[d]	NT
2		31.3 ± 1.8 (49.6)	NT
3		16.0 ± 0.4 (32.5)	9.4 ± 2.6 (53.2)
4	8	36.0 ± 1.1. (47.7)	17.2 ± 5.2 (42.5)
5		8.9 ± 2.0 (42.5)	NT

a DA → AO: AO recipient of DA skin grafts.

b Draining lymph nodes (axillary and brachial)

c $\left(100 \times \frac{(I - N)}{(M - N)}\right)$ mean ± S.E. of triplicates. 18 hour assay. The lymphocyte/target cell ratio was 100:1

d Per cent ^{51}Cr-release in the presence of non-immune cells is given in parentheses.

background release after a less prolonged culture period, an experiment was carried out in which immune cells were incubated with ^{51}Cr-labelled fibroblasts for either 7 hours or 18 hours. The results are presented in TABLE 2. Specific ^{51}Cr-release was significantly greater after 18 hours. Background release of ^{51}Cr was high (30-40%) even after 7 hours and was only slightly greater after 18 hours.

(b) ^{51}Cr-labelled lymphoma cells as target cells.

The C58NTD Gross-virus induced lymphoma of Wistar Furth rats has been used as a target cell for CMC in a rat syngeneic tumour system (Oren et al. 1971). A cell line established from this tumour, the WF/G_1 lymphoma, was tested as a target cell in this system. The lymphoma cells express the major histocompatibility antigens of the parent strain (Ag-B2) and can therefore be used as a target cell for cytotoxic lymphocytes with specificity for this antigen complex.

The results of an experiment in which DA rats were immunised with either WF or AO skin grafts and the draining lymph node cells were tested for cytotoxic activity against ^{51}Cr-labelled WF/G_1 lymphoma cells are presented in TABLE 3. In both strain combinations, significant cytotoxicity was measured at lymphocyte/target cell ratios of 300:1 and 100:1. The spontaneous release and the background release of ^{51}Cr from the lymphoma cells was 37.8 and 27.0% respectively, after 8 hours in culture.

Both the AO and WF rat strains are Ag-B2 and in this experiment lymphocytes from DA rats (Ag-B4) immunised with AO skin grafts were at least as cytotoxic for the WF/G_1 lymphoma cells as were lymphocytes from DA rats immunised with WF grafts (i.e. of the same phenotype as the target cells).

The cytotoxicity measured in this experiment was sufficiently high to justify a more extended series of experiments using

TABLE 3.2

CYTOTOXICITY AGAINST ^{51}Cr-LABELLED FIBROBLASTS

IMMUNE LYMPHOCYTES [a]	PER CENT SPECIFIC ^{51}Cr-RELEASE [b] 7 hrs	18 hrs
SPLEEN	10.7 ± 4.4 (33.5)[c]	18.1 ± 4.8 (44.0)
LYMPH NODES	16.6 ± 4.2 (38.5)	33.2 ± 1.3 (45.2)

a DA → AO. 8 days after grafting. Lymphocyte/target cell ratio 100:1

b $\left\{100 \times \left(\frac{I}{M} - \frac{N}{M}\right)\right.$ mean ± S.E. of triplicates.

c Per cent ^{51}Cr-release in presence of non-immune lymphocytes

TABLE 3.3

CYTOTOXICITY ACAINST ^{51}Cr-LABELLED WF/G_1 LYMPHOMA CELLS

STRAIN COMBINATION [a]	TARGET [b]	LYMPHOCYTE/TARGET CELL RATIO [c]	PER CENT SPECIFIC ^{51}Cr-RELEASE [d]
WF → DA	WF/G_1	300:1	28.0 ± 0.7
		100:1	6.4 ± 1.4
AO → DA		300:1	38.5 ± 2.6
		100:1	11.8 ± 1.4

a WF → DA; DA recipient of WF skin graft.

b 1×10^5 target cells.

c Draining lymph node cells, 7 days after grafting.

d 8 hour assay. $\left(100 \times \left(\frac{I}{M} - \frac{N}{M}\right)\right)$ mean ± S.E. of triplicates. Background release was 27%

^{51}Cr-labelled WF/G_1 target cells. The results obtained in nine separate experiments in which lymphocytes from DA recipients of AO skin grafts were tested for cytotoxicity against WF/G_1 target cells are presented in TABLE 4. Specific ^{51}Cr-release was generally low and there was considerable variation between different experiments. The lymphocyte/target cell ratio required to measure significant specific ^{51}Cr-release was high (200:1) and the assay was not sufficiently sensitive to detect a satisfactory dose/response.

(c) ^{125}IUdR-labelled lymphoma cells as target cells.

The experiments described in the preceding sections showed ^{51}Cr to be unsuitable as a label for either fibroblasts or the lymphoma cell line under the conditions of assay employed; spontaneous release of the isotope from both cell types was unacceptably high even after short incubation periods. The WF/G_1 lymphoma grows rapidly in vitro with a doubling-time of 12-15 hours under optimal culture conditions, which suggested that these cells might be suitable for pre-labelling in vitro with a radioactive DNA precursor. Target cells labelled with ^{125}Iododeoxyuridine (^{125}IUdR) have been used in assays of cell-mediated cytotoxicity (Oldham and Herberman 1973). A preliminary study (C. Haslett in this laboratory), in which the optimal conditions for labelling the WF/G_1 lymphoma cells with ^{125}IUdR were established, showed that sufficient isotope was incorporated within the cells in 24 hours and that the spontaneous release of ^{125}I from the cells was low (10-20% at 18 hours). The results of an experiment in which the cytotoxic activity of lymph node cells from DA rats immunised with AO skin grafts was assayed against WF/G_1 lymphoma cells labelled with either ^{51}Cr or ^{125}IUdR are presented in TABLE 5. The ^{51}Cr-labelled cells were incubated with immune lymphocytes for 5 hours and the ^{125}IUdR-labelled cells for either 5 or 18 hours. Low

TABLE 3.4

CYTOTOXICITY AGAINST ^{51}CR-LABELLED WF/G_1 LYMPHOMA CELLS

EXPT [a]	PER CENT SPECIFIC ^{51}Cr-RELEASE [c]		
	200:1 [b]	100:1	50:1
1	18.8 ± 0.9	5.0 ± 0.4	
2	21.1 ± 0.6	8.5 ± 2.7	
3	9.9 ± 1.6	5.2 ± 1.4	
4	4.7 ± 0.6	1.8 ± 0.2	
5	10.6 ± 0.8		1.9 ± 0.6
6	9.7 ± 0.4		2.3 ± 0.9
7	4.1 ± 0.2		2.3 ± 0.4
8	8.7 ± 3.9		1.1 ± 1.0
9	5.1 ± 0.6		3.6 ± 0.5

a In all expts. immune lymphocytes were lymph node cells obtained 7 days after skin grafting.

b Lymphocyte/target cell ratio. 1×10^5 WF/G_1 target cells.

c 5 hour assay $\left(100 \times \frac{I}{M} - \frac{N}{M}\right)$ mean ± S.E. of triplicates.
Background release was 22%

TABLE 3.5

A COMPARISON OF CYTOTOXICITY AGAINST ^{51}Cr- AND ^{125}I-LABELLED WF/G_1 TARGET CELLS

STRAIN COMBINATION	TARGET	LYMPHOCYTE/ TARGET CELL RATIO [a]	PER CENT SPECIFIC ISOTOPE RELEASE [b] ^{51}Cr-(5 hrs)	^{125}I-(5 hrs)	^{125}I-(18 hrs)
AO → DA	WF/G_1	200:1	10.1 ± 0.8	7.2 ± 0.2	32.1 ± 0.3
		50:1	2.4 ± 0.5	2.3 ± 0.4	8.9 ± 1.5

a Draining lymph node cells obtained 7 days after skin grafting.

b $\left(100 \times \left(\frac{I}{M} - \frac{N}{M}\right)\right)$ mean ± S.E. Background release: ^{51}Cr; 16.0% (5 hrs), ^{125}I; 21.2% (5 hrs), 33.4% (18 hrs)

levels of cytotoxicity against both ^{51}Cr and ^{125}IUdR-labelled cells were detected after 5 hours. Cytotoxic activity against the ^{125}IUdR-labelled cells was substantially increased after 18 hours. A further series of experiments was carried out using ^{125}IUdR-labelled WF/G_1 target cells, and the results are presented in TABLE 6. Significant cytotoxicity was detected over a wide range of lymphocyte/target cell ratios and there was a near linear relationship between ^{125}I-release and the logarithm of immune cell number (Fig. 1). These experiments also showed that the reaction was specific for the immunising antigens; lymphocytes from rats immunised with HO (Ag-B5) skin grafts were not cytotoxic for the WF/G_1 target cells.

The cytotoxic assays described so far were carried out in 1 ml. volumes in LINBRO tissue-culture plates using 1×10^5 labelled target cells per well and lymphocyte/target cell ratios of from 400:1 to 50:1. Because of the obvious advantages of a smaller scale assay system, experiments were carried out in which immune cells were incubated with the target cells in 0.2 ml. volume in microtitre plates. The target cell number was reduced to 2.5×10^4 cells/well and cytotoxicity was measured at lymphocyte/target cell ratios of 200:1 to 6.25:1. The results obtained in four separate experiments are presented in TABLE 7. In both strain combinations tested, significant levels of cytotoxicity were measured at lymphocyte/target cell ratios of from 100:1 to 6.25:1, and ^{125}I-release was proportional to the logarithm of immune cell number. The background release of isotope in the presence of non-immune lymphocytes was 10% at 18 hours. The reduction in cytotoxicity at high lymphocyte/target cell ratios (Expts. 1 and 4, TABLE 7) was a fairly consistent feature and may have been due to overcrowding at high cell numbers/well.

This assay system, in which ^{125}IUdR-labelled WF/G_1 lymphoma target cells were incubated with immune lymphocytes in microtitre plates for

TABLE 3.6

CYTOTOXICITY AGAINST ^{125}IUdR-LABELLED TARGET CELLS

IMMUNE LYMPHOCYTES [a]	LYMPHOCYTE/ TARGET CELL RATIO [c]	PER CENT SPECIFIC ^{125}I RELEASE [d] EXPT 1.	EXPT 2.	EXPT 3. ℓ
AO → DA [b]	400:1	49.1 ± 3.2	31.2 ± 1.5	36.7 ± 0.8
	200:1	31.8 ± 1.2	22.6 ± 0.8	23.9 ± 3.3
	100:1	10.6 ± 1.5	9.5 ± 1.9	15.6 ± 3.2
	50:1	5.5 ± 2.4	2.6 ± 1.0	3.5 ± 2.6
HO → DA	400:1	< 1.0%	< 1.0%	1.1 ± 0.4

a Lymph node cells obtained 7 days after grafting.

b AO → DA; DA recipient of AO skin graft.

c 1×10^5 WF/G_1 lymphoma cells.

d 18 hour assay: $\left(100 \times \frac{I}{M} - \frac{N}{M}\right)$ mean ± S.E. of triplicates. The mean background release was 32%

ℓ Experiment 3 is illustrated in Fig. 1.

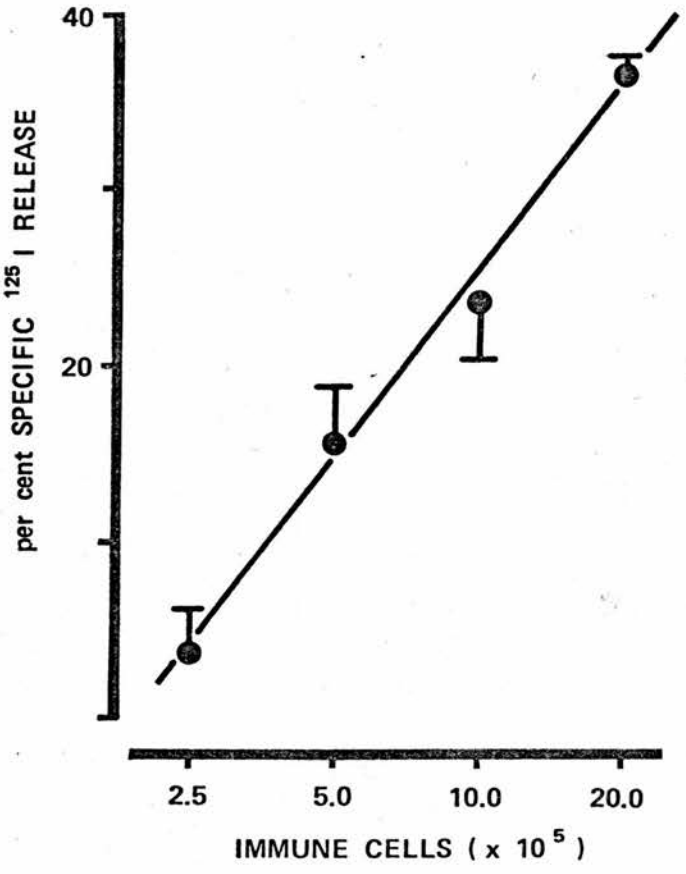

Fig. 3.1. The relationship between specific ^{125}I-release and immune cell number.

Different numbers of immune cells obtained from the draining lymph nodes of skin allografted rats were incubated with 2 x 10^4 WF/G_1 target cells labelled with ^{125}IUdR for 18 hours. Each point represents the mean ± S.E. of triplicate cultures.

TABLE 3.7

CYTOTOXICITY AGAINST ^{125}IUdR-LABELLED TARGET CELLS: MICROTITRE PLATE ASSAY

LYMPHOCYTE/ TARGET CELL RATIO [b]	PER CENT SPECIFIC ^{125}I-RELEASE [c]			
	EXPT 1. AO → DA	EXPT 2. AO → DA	EXPT 3. AO → HO	EXPT 4. AO → HO
200:1	26.7 ± 1.1	41.3 ± 5.4	NT	8.3 ± 0.9
100:1	33.0 ± 2.3	38.7 ± 1.4	44.7 ± 2.3	17.8 ± 1.9
50:1	29.8 ± 1.9	31.0 ± 0.9	34.1 ± 1.0	21.9 ± 0.9
25:1	26.6 ± 2.7	30.0 ± 1.1	29.2 ± 2.3	16.9 ± 2.0
12.5:1	12.9 ± 1.1	22.8 ± 4.4	21.6 ± 1.5	11.1 ± 1.3
6.25:1	8.2 ± 1.3	NT	15.9 ± 1.4	8.0 ± 1.3

a Draining lymph node cells obtained 8 days after skin grafting.

b 2.5×10^4 WF/G_1 lymphoma cells

c 18 hour assay: $\left(100 \times \frac{I - N}{M \quad M}\right)$ mean ± S.E. triplicates. Background release was 10%

18 hours, was the most satisfactory and was used in all subsequent experiments.

It has been reported that treatment of CMC cultures with trypsin at the end of the incubation period increased the amount of isotope released from target cells already damaged in the cytotoxic reaction (Vaino et al. 1964, Oldham and Herberman 1973). Experiments were carried out to investigate the possibility that specific ^{125}I-release in this system might be enhanced in similar fashion by treatment of the microcultures with the enzyme DNAase (TABLE 8). Enzyme treatment actually reduced specific ^{125}I-release at high lymphocyte/target cell ratios, and the effect at lower ratios was variable. The linear relationship between immune cell number and specific ^{125}I-release was distorted. The background release of ^{125}I- was increased from 12 to 20% after incubation with the enzyme.

3.2 The Specificity of the Cytotoxic Response.

The experiments presented in TABLE 6 showed that the cytotoxic response to skin allografts was specific at the induction phase of the response; lymphocytes from rats immunised with HO (Ag-B5) skin grafts were not cytotoxic for the WF/G_1 (Ag-B2) target cells. Because the WF/G_1 was the only tumour target cell available, it was not possible to examine the specificity of the response at the effector cell level using the more sensitive ^{125}I-release assay. However, it was possible to test this in experiments which used ^{51}Cr-labelled fibroblasts of different genotypes as target cells. The results of two experiments in which AO rats were immunised with either DA or HO skin grafts and the draining lymph node cells were tested for cytotoxicity activity against both DA and HO fibroblasts are presented in TABLE 9. Cytotoxic activity against the target cells expressing AgB antigens unrelated to

TABLE 3.8

THE EFFECT OF DNAase[a] ON SPECIFIC ^{125}I-RELEASE

IMMUNE[b] LYMPHOCYTES ($x10^5$)	LYMPHOCYTE/ TARGET CELL RATIO[c]	PER CENT SPECIFIC ^{125}I-RELEASE[d] EXPT. 1		EXPT. 2	
		NO ENZYME	DNAase	NO ENZYME	DNAase
50.0	200:1	36.5 ± 6.1	12.0 ± 3.0	30.1 ± 1.3	15.0 ± 2.2
25.0	100:1	34.4 ± 0.5	10.9 ± 0.3	37.1 ± 1.4	24.1 ± 2.2
12.5	50:1	25.3 ± 0.3	24.0 ± 1.0	33.7 ± 0.2	20.5 ± 1.1
6.25	25:1	16.4 ± 0.5	32.4 ± 6.6	30.4 ± 1.2	16.0 ± 1.5
3.2	12.5:1	11.5 ± 0.9	17.8 ± 3.5	21.2 ± 0.9	18.3 ± 2.2
1.6	6.25:1	8.4 ± 0.7	13.3 ± 0.8	16.6 ± 0.1	19.7 ± 1.7

a 10 µl DNAase (1 mg/ml) was added to each culture and the plates were incubated at 37°C for 30 minutes prior to centrifugation and removal of the supernatant medium.

b AO → DA. Lymph node cells obtained 8 days after grafting.

c 2.5×10^4 WF/G_1 lymphoma cells.

d $\left(100 \times \frac{I - N}{M - N}\right)$ mean ± S.E. triplicates.

TABLE 3.9

THE SPECIFICITY OF THE CYTOTOXIC RESPONSE

EXPT.	STRAIN COMBINATION [a]	TARGET [b]	PER CENT SPECIFIC ^{51}Cr-RELEASE [c]
1	DA → AO	DA	31.3 ± 1.8
		HO	< 1.0
	HO → AO	HO	11.6 ± 1.7
		DA	3.2 ± 2.3
2	DA → AO	DA	16.0 ± 0.4
		HO	< 1.0
	HO → AO	HO	12.8 ± 2.2
		DA	2.7 ± 1.5

a DA → AO, AO recipient of DA skin graft. Draining lymph node cells 7 days after grafting.

b 1×10^5 fibroblasts. The lymphocyte/target cell ratio was 100:1

c $\left(100 \times \left(\frac{I}{M} - \frac{N}{M}\right)\right)$ mean $\pm$ S.E. triplicates

the immunising antigens was negative in the DA → AO strain combination and was not statistically significant in the HO → AO strain combination.

3.3 The Kinetics of the Response.

The kinetics of the cytotoxic response in the draining lymph nodes of skin allografted rats was investigated and the results of a representative experiment are presented in TABLE 10. Cell-mediated cytotoxicity was detectable six days after grafting, was maximal on day 8 and declined rapidly thereafter to a level just above background on day 10. Similar results were obtained in two other experiments. In view of these results, all subsequent experiments used lymph node cells taken 8 days after grafting.

3.4 The Nature of the Cytotoxic Cell.

The term cell-mediated cytotoxicity has been applied to a number of different lytic reactions *in vitro* which involve the participation of lymphoid or non-lymphoid cells from normal or immune donors, but which are complement independent. There are a number of different mechanisms involved and these reactions involve different effector cells according to the *in vitro* models studied. Three main categories have been described;

(1) T-cell mediated cytotoxicity, in which thymus-derived lymphocytes from immunised donors are specifically cytotoxic for target cells which express the immunising antigens on their surface (Cerottini et al. 1970).

(2) K-cell mediated, or antibody-dependent cytotoxicity (ADC), in which normal lymphoid cells are cytotoxic against antibody-coated target cells (Perlmann and Holm 1969). The nature of the effector cell is still controversial but T-lymphocytes are not involved (Van Boxel et al. 1972, Harding et al. 1971).

TABLE 3.10

THE KINETICS OF THE CYTOTOXIC RESPONSE AFTER SKIN GRAFTING

IMMUNE [a] LYMPHOCYTES ($x10^5$)	LYMPHOCYTE/ TARGET CELL RATIO[b]	PER CENT SPECIFIC ^{125}I-RELEASE [c] DAYS AFTER GRAFTING				
		6	7	8	9	10
25.0	100:1	11.5 ± 0.7	17.6 ± 1.7	24.9 ± 0.5	18.8 ± 1.5	4.2 ± 0.3
12.5	50:1	11.9 ± 0.3	16.6 ± 2.0	18.9 ± 0.7	13.3 ± 0.8	4.6 ± 0.3
6.25	25:1	11.8 ± 2.0	11.6 ± 1.9	11.9 ± 0.9	8.0 ± 1.4	1.9 ± 0.2
3.2	12.5:1	8.4 ± 0.8	6.9 ± 0.6	6.1 ± 0.7	5.5 ± 0.5	< 1.0

a Draining lymph node cells from DA recipients of AO skin grafts.

b 2.5×10^4 WF/G_1 target cells.

c $\left(100 \times \frac{I - N}{M - N}\right)$ mean ± S.E. of triplicates.

(3) Macrophage-mediated cytotoxicity, in which macrophages become specifically cytotoxic ("armed") by contact with either immune lymphoid cells or with cell-free supernatants from cultures of immune cells incubated with antigen (Evans and Alexander 1970, 1971).

Although the relative importance of these different reactions in different experimental systems in vivo has yet to be established, it has been demonstrated by a number of investigators that the cell-mediated cytotoxic response to both tumour allografts and skin allografts in the mouse (Lonai et al. 1971, Golstein et al. 1972) and the rat (Peter and Feldman 1972, Tucker et al. 1974) is mediated by specifically sensitised T-cells; the cytotoxic activity of immune cell populations was completely removed by treatment with antisera specific for T-cells and complement.

The nature of the effector cell in the cytotoxic reaction described here was investigated by indirect methods:

(1) Effector cell populations, obtained from the lymph nodes of skin allografted rats, were depleted of B-cells by passage through anti-Ig coated plastic bead columns and were tested for cytotoxic activity. The results obtained in two experiments are presented in TABLE 11. Passage through the anti-Ig columns resulted in a 95% depletion of immunoglobulin-positive (Ig^+) lymphocytes, as judged by indirect immunofluorescence. The cytotoxic activity of the anti-Ig column-passed cells was the same as the activity of cells passaged through control columns (20-30% depleted of Ig^+ cells). The activity of both column-passed cell populations was slightly reduced compared to unfractionated cells; this was probably a consequence of the extra handling to which the cells had been subjected.

(2) The cytotoxic activity of immune cell populations depleted of and enriched in F_c receptor-bearing cells by incubation on antibody-coated sheep erythrocyte monolayers (Kedar et al. 1974b) was compared in two

TABLE 3.11

CYTOTOXIC ACTIVITY OF LYMPHOCYTES[a] AFTER PASSAGE THROUGH ANTIBODY-COATED COLUMNS

EXPT.	TYPE OF COLUMN	FLUORESCENT[c] (%)	PER CENT SPECIFIC ^{125}I-RELEASE[d] 100:1	50:1	25:1
	--------	35.0	30.4 ± 0.4	19.1 ± 1.8	11.8 ± 2.0
1	Ig-anti Ig	1.6	22.5 ± 1.2	14.4 ± 0.4	1.7 ± 0.5
	Normal serum[b]	24.0	24.9 ± 0.6	13.5 ± 0.5	2.8 ± 0.6
	--------	40.0	32.2 ± 2.4	26.8 ± 0.7	8.5 ± 0.8
2	Ig-anti Ig	2.4	19.5 ± 2.0	11.2 ± 0.5	4.8 ± 0.5
	Normal serum	31.0	18.2 ± 1.0	11.3 ± 0.8	5.5 ± 0.4

a Draining lymph node cells obtained 8 days after skin grafting.

b Normal rabbit serum was substituted for rabbit anti-rat Ig serum.

c Air dried smears were fixed in cold acetone (-20°C) exposing both intracellular and surface Ig to fluorescent staining.

d $\left(100 \times \frac{I - N}{M - N}\right)$ mean ± S.E.

TABLE 3.12

CYTOTOXIC ACTIVITY OF LYMPHOCYTE POPULATIONS AFTER INCUBATION ON ANTIBODY-COATED CELL MONOLAYERS [a]

EXPT.	CELLS [b]	FLUORESCENT (%)	PER CENT SPECIFIC ^{125}I-RELEASE [c] 100:1	50:1	25:1
1	UNFRACTIONATED	35.0	20.0 ± 1.8	16.0 ± 1.4	8.1 ± 2.8
	NON-ADHERENT	3.0	20.1 ± 3.5	18.7 ± 2.8	9.2 ± 1.3
	ADHERENT	>99.0	< 1.0	< 1.0	<1.0
2	UNFRACTIONATED	37.0	25.0 ± 0.4	19.2 ± 0.8	14.3 ± 1.4
	NON-ADHERENT	2.0	26.4 ± 0.5	21.0 ± 1.0	16.0 ± 0.3
	ADHERENT	>98.0	3.2 ± 1.0	2.5 ± 1.0	<1.0

a SRBC monolayers coated with rabbit anti-SRBC antiserum.

b Draining lymph node cells obtained 8 days after skin allografting.

c $\left(100 \times \frac{I - N}{M - N}\right)$ mean ± S.E. of triplicates.

TABLE 3.13

THE IMPORTANCE OF Ag-B AND NON-Ag-B ANTIGENS IN THE CYTOTOXIC RESPONSE

IMMUNE LYMPHOCYTES [a] ($x10^5$)	LYMPHOCYTE/ TARGET CELL RATIO [b]	PER CENT SPECIFIC ^{125}I-RELEASE [d]					
		EXPT. 1			EXPT. 2		
		WF → HO [c]	HO.B2 → HO	WF → HO.B2	WF → HO	HO.B2 → HO	
25.0	100:1	25.9 ± 1.5	30.7 ± 1.1	5.9 ± 0.5	28.4 ± 0.9	42.1 ± 1.5	
12.5	50:1	23.0 ± 1.2	26.0 ± 0.6	8.9 ± 0.8	29.0 ± 2.9	37.3 ± 3.0	
6.25	25:1	13.9 ± 0.5	18.0 ± 0.6	6.0 ± 1.0	26.0 ± 2.3	31.3 ± 1.9	
3.175	12.5:1	10.3 ± 1.1	10.7 ± 0.9	3.9 ± 0.2	24.1 ± 1.4	23.4 ± 0.6	
1.5875	6.25:1	5.9 ± 0.3	5.3 ± 0.8	2.3 ± 0.9	19.4 ± 1.9	18.6 ± 1.6	

a Draining lymph node cells 8 days after grafting.

b 2.5×10^4 WF/G_1 cells.

c HO recipient of WF skin graft.

d $\left(100 \times \frac{I - N}{M - N}\right)$ mean ± S.E. triplicates.

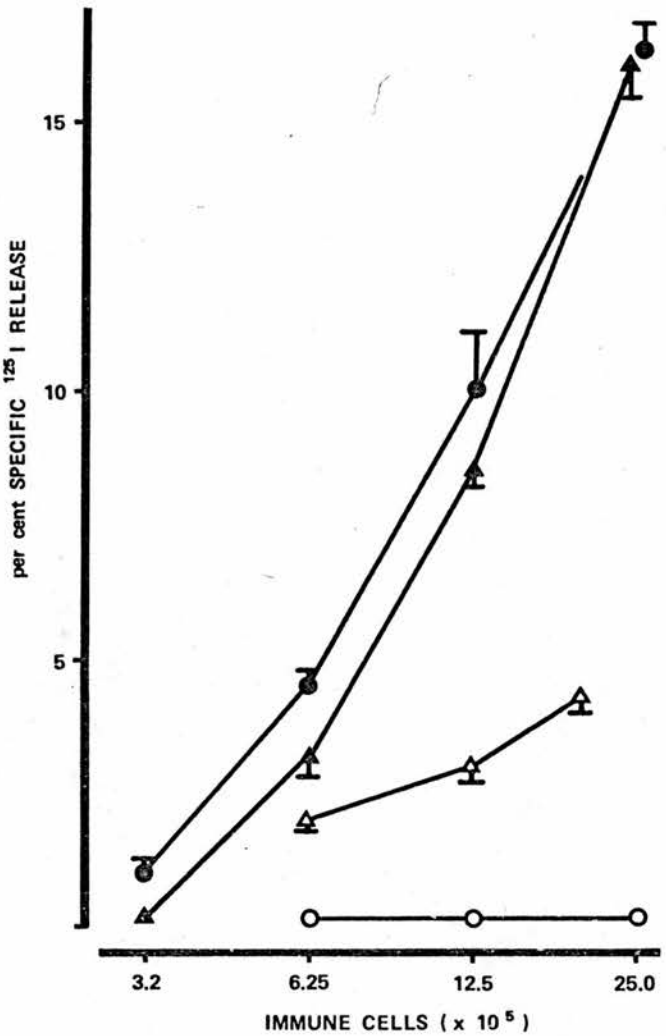

Fig. 3.2 The contribution of AgB and non-AgB alloantigens to the cytotoxic response.

Draining lymph node cells were obtained 8 days after skin allografting in four different strain combinations and were tested for cytotoxic activity against ^{125}IUdR-labelled WF/G_1 target cells. Donors and recipients differed in AgB antigens alone (HO.B2 → HO, ●——●); in AgB and non-AgB antigens (WF → HO, ▲——▲); and in non-AgB antigens alone (WF → HO.B2, △——△ ; WF → AO, ○——○).

separate experiments (TABLE 12). The cytotoxic activity of the non-adherent cells was the same as the activity of the unfractionated cells despite a > 90% depletion of Ig^+ cells. The adherent cell populations, which comprised > 98% Ig^+ cells, were more than 95% depleted in cytotoxic activity.

3.5 The Contribution of AgB antigens to the cytotoxic response.

In all the experiments described so far, the donor and recipients of skin grafts differed in alloantigens determined by the major histocompatibility locus AgB. The relative contribution of AgB and non-AgB alloantigens to the cytotoxic response was investigated by skin grafting in strain combinations in which the donor and recipient differed in AgB antigens alone (HO.B2 → HO), in both AgB and non-AgB antigens (WF → HO) and in non-AgB antigens alone (WF → HO.B2, WF → AO). In three separate experiments the cytotoxic response in strain combinations differing in AgB antigens alone was at least as strong as in combinations differing in both AgB and non-AgB antigens (TABLE 13, Fig. 3). The response in the non-AgB different strain combination WF → HO.B2 was reduced by approximately three-fold compared to the response in AgB-different combinations, and in the strain combination WF → AO no cytotoxicity was detected (Fig. 3).

DISCUSSION

A sensitive, reproducible and quantitative isotope-release assay for measuring the cell-mediated cytotoxic response to allogeneic skin grafts is described. The target cell/isotope combination which was found to be the most satisfactory was the WF/G_1 lymphoma cell line labelled with ^{125}IUdR. ^{51}Cr-labelled fibroblasts and WF/G_1 cells were found to be unsatisfactory target cells in this system because of the

unacceptably high spontaneous release of ^{51}Cr from these cells within the first few hours of culture. Significant cytotoxic activity was measured over a wide range of lymphocyte/target cell ratios using the $^{125}IUdR$-labelled lymphoma target cells and there was a quantitative relationship between specific ^{125}I-release and the logarithm of immune cell number. A similar relationship has been described by others (Canty and Wunderlich 1971, MacDonald et al. 1973) in mouse allograft systems. At the present time there is no simple assay procedure available for the detection of individual cytotoxic effector cells. If, however, a sufficiently sensitive assay system is available, empirical estimates of the relative frequency of effector cells in different populations can be made by exploiting this relationship between isotope-release and the concentration of effector cells (MacDonald et al. 1973). The assay described here should therefore permit accurate comparisons of the cytotoxic activity of different lymphocyte populations (Ch.4). The use of DNAase to increase the sensitivity of the assay appeared to beunnecessary since in no case did enzyme treatment detect cytotoxicity where the total ^{125}I-release was not already significantly different from the controls.

The cytotoxic response after skin grafting was specific at both the induction and the effector phase of the response. Lymphocytes from rats immunised with AgB antigens unrelated to those expressed on the WF/G_1 lymphoma cells were not cytotoxic for these cells and sensitised lymphocytes were not cytotoxic for target cells bearing alloantigens unrelated to those of the skin graft.

Peter and Feldman (1972) reported that the cytotoxic activity of lymphocytes from skin allografted rats was abolished after incubation with an anti-T cell serum and complement and others have described

similar findings in a rat tumour allograft system (Tucker et al. 1974). The cell-mediated cytotoxic response to skin grafts is maximal at a time when the alloantibody response in these rat strains is just detectable and is negative at the time of peak antibody production (Rolstad et al. 1974). The experiments presented here showed that depletion of B-cells from the effector cell population had no effect on the cell-mediated cytotoxicity, and that cell populations enriched in B-cells (>98% Ig^+ lymphocytes) were not cytotoxic under the conditions of assay employed. These findings argue against the participation of B-cells or alloantibody in the cytotoxic reaction described here and suggest that the cytotoxic cell is a thymus-derived (T) lymphocyte, as has been found to be the case in a number of allograft systems.

There are a number of recent reports in the literature which suggest that at least a proportion of T-cells activated to histocompatibility antigens have F_c receptors (e.g. Yoshida and Andersson 1972, Basten et al. 1975). There was no evidence from the experiments reported here that the cytotoxic cells possessed these receptors; cell populations adherent to antibody-coated sheep erythrocyte monolayers were not cytotoxic.

Although the T-cell mediated cytotoxic reaction has been widely accepted as an *in vitro* model of graft rejection , the exact role of cytotoxic T-cells in allograft destruction *in vivo* has been difficult to establish. Cytotoxic T-cell populations cause accelerated skin graft rejection in neonatally thymectomised mice (Sprent and Miller 1972) and there is recent evidence in both the mouse (Lance and Cooper 1972) and the rat (Tilney and Ford 1974) that immune cell populations, obtained from skin allografted animals, accumulate preferentially at the site of specific allografts on transfer to syngeneic skin grafted

recipients. Tilney et al. (1975) have also recently shown that cytotoxic T-cells could be recovered from acutely rejecting cardiac allografts in the rat. Although these results demonstrated that the cellular infiltrate in a rejecting allograft included cytotoxic T-cells, it has not yet been established whether or not these cells are solely responsible for the destruction of the graft. Many of the mononuclear cells in a rejecting skin allograft are of recent bone-marrow origin and probably belong to the monocyte or macrophage series (Giroud et al. 1970). The demonstration that macrophages can become specifically cytotoxic in the presence of immune lymphoid cells and antigen (Evans and Alexander 1970) make it an attractive possibility that the encounter of cytotoxic T-cells with antigen in the graft results in an amplification of the cytotoxic effector cell response by specifically "armed" cytotoxic macrophages. However it remains an open question as to whether the macrophages play an active part in graft destruction or merely scavenge dead or dying cells.

Skin grafting between congenic rat strains, differing only at the major histocompatibility complex (AgB), stimulated cytotoxic responses which were as great as the responses measured in strain combinations where donor and recipient differed in both AgB and non-AgB alloantigens. Skin grafting between strains which differed only in non-AgB alloantigens stimulated little or no cytotoxic response. These data suggest that non-AgB alloantigens do not contribute significantly to the cell-mediated cytotoxic response to allogeneic skin grafts, either when presented alone or in combination with AgB alloantigens. This is in agreement with the findings of others on the relative contribution of H-2 and non H-2 alloantigens to cell-mediated cytotoxic responses in mice (Festenstein 1973).

CHAPTER FOUR

THE ADHERENCE OF CYTOTOXIC LYMPHOCYTES TO ALLOGENEIC CELL MONOLAYERS.

Since the demonstration by Brondz et al. (1968, 1970) that immune lymphoid cells could be depleted of cytotoxic activity by incubation on cell monolayers of the appropriate immunising specificities, monolayers of a variety of cell types have been used as immuno-adsorbents for the cytotoxic lymphocytes produced as a consequence of allogeneic immunisation. Specific depletion of cytotoxic cells has been accomplished by adsorption on monolayers of macrophages (Brondz 1968), fibroblasts (Berke and Levey 1972, Golstein et al. 1971), tumour cells (Stulting and Berke 1973) and normal spleen cells (Bonavida and Kedar 1974). Lymphocytes non-adherent to monolayers of the immunising specificities were shown to have negative or reduced cytotoxic activity towards target cells syngeneic to the monolayer cells, whereas cells non-adherent to monolayers expressing unrelated antigens were not depleted of cytotoxic activity. Direct evidence that the reduction in the cytotoxic activity of the non-adherent cell populations was a consequence of the specific adsorption of cytotoxic lymphocytes on the relevant cell monolayers was obtained in experiments in which the adherent cells were recovered from the monolayers by trypsinisation (Golstein et al. 1971), or by treatment with EDTA (Stulting and Berke 1973) and were found to be cytotoxic.

The experiments in this chapter show that thoracic-duct lymphocyte monolayers are also effective immuno-adsorbents for cytotoxic lymphocytes. The adherence of cytotoxic lymphocytes to these monolayers was immunologically specific, and conditions of adsorption were found under which not only were the cell populations non-adherent to specific allogeneic monolayers substantially depleted of cytotoxic cells, but the adherent cells recovered from the monolayers by treatment with EDTA were substantially enriched in cytotoxic cells.

RESULTS

4.1. Depletion and enrichment of cytotoxicity after incubation of lymphocyte populations on allogeneic lymphocyte monolayers.

Cytotoxic cell populations were obtained from the draining lymph nodes of skin grafted rats, 8 days after grafting. In all the experiments described the donors and recipients of skin grafts differed at the major histocompatibility locus AgB. Cytotoxic cell populations were incubated on monolayers of the same strain as the immunising antigens, and the non-adherent and adherent cell populations were assayed for cytotoxic activity against ^{125}IUdR-labelled WF/G_1 lymphoma cells. The results obtained in four separate experiments are illustrated in Figs. 1a-1d. In each experiment the cytotoxic activity of the non-adherent cell populations was substantially reduced compared to the unfractionated cells and the activity of the adherent cells was significantly increased. The dose response curves obtained for the different cell populations in each experiment were parallel or very nearly so, at least at intermediate lymphocyte/target cell ratios. The relative activity of the different lymphocyte populations could therefore be accurately quantitated by comparing the numbers of non-adherent and adherent cells required to produce the same cytotoxic response as the control (unfractionated) cell population. This was computed from the horizontal distance between the lines of best fit over the linear portions of the curves, as described by Michie (1973), and the results of seven adsorption experiments, including the four illustrated in Fig. 1, are summarised in TABLE 1. The cytotoxic activity of the non-adherent and adherent cells, compared to the unfractionated cells, is expressed as a potency ratio; ratios greater than unity represent an increase in cytotoxic activity and ratios less

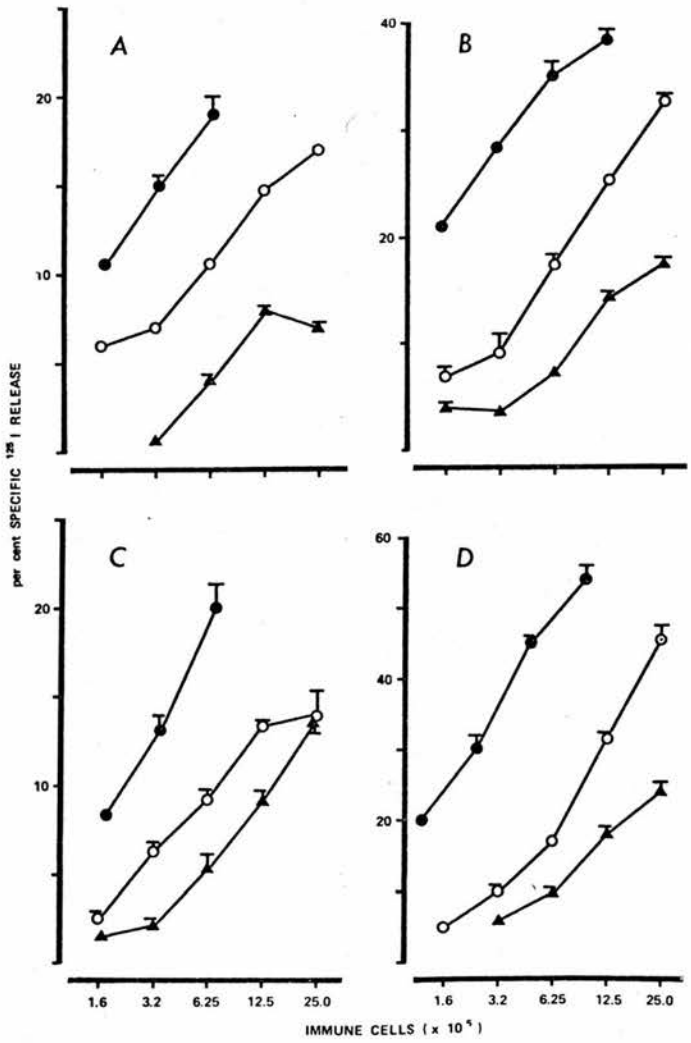

Fig 4.1 The cytotoxic activity of lymphocytes adherent and non-adherent to specific allogeneic lymphocyte monolayers.

Lymph node cells from HO rats immunised with AO skin grafts were incubated on AO.TDL monolayers and were tested for cytotoxic activity against WF/G_1 target cells in four separate experiments (a-d). Unfractionated cells ○——○ ; adherent cells ●——● non-adherent cells ▲——▲ . Statistical comparison of the activity of the different cell populations was by analysis of co-variance. There was no significant difference in the slope of the regression lines calculated for the different cell populations in any experiment ($p > 0.10$). In each experiment the activity of the adherent and non-adherent cells was significantly different from the unfractionated cells ($p < 0.025$).

TABLE 4.1

DEPLETION AND ENRICHMENT OF CYTOTOXIC LYMPHOCYTES AFTER INCUBATION ON SPECIFIC ALLOGENEIC LYMPHOCYTE MONOLAYERS [a]

	RELATIVE CYTOTOXIC ACTIVITY [b]	
EXPT.	NON-ADHERENT CELLS	ADHERENT CELLS
1	0.56	1.54
2	0.42	1.83
3	0.40	1.96
4	0.53	4.77
5	0.55	5.23
6	0.48	2.94
7	0.20	3.20
MEAN ± s.e.	0.45 ± 0.05	3.06 ± 0.55

a Lymph node cells from HO rats immunised with AO skin grafts were incubated on AO.TDL monolayers.

b Compared to unfractionated cells. Relative activity was calculated by a parallel line assay as described in the text. Expts. 4-7 are illustrated in Figs. 1a-d respectively.

than unity a reduction in activity. In each experiment the reduction in activity in the non-adherent cell populations was accompanied by an increase in the activity of the adherent cells recovered from the monolayers: the non-adherent cells were 45-80% depleted in cytotoxic activity and there was a 1.5 to 5-fold enrichment in activity in the adherent cell populations.

Since the monolayers used as immuno-adsorbents for cytotoxic lymphocytes are of necessity of target cell specificity, it was important to establish whether or not significant numbers of TDL became detached from the monolayers during the adsorption procedure, since these cells might be expected to compete with the labelled lymphoma target cells in the cytotoxic assay. This would result in differences in the observed cytotoxic activity (per cent ^{125}I-release) of the different cell populations which would not necessarily be directly related to changes in the numbers of cytotoxic cells present. The numbers of monolayer cells which became detached during the adsorption procedure was determined in experiments in which normal lymphocytes were incubated on monolayers prepared from ^{3}H-uridine or ^{3}H-leucine-labelled TDL. The non-adherent and adherent cells were recovered in the usual way. Smears of the different cell populations were prepared, and the numbers of labelled cells present in each fraction was assessed by autoradiography. Monolayer cells accounted for 3% of the non-adherent and 15% of the adherent cell populations (TABLE 2).

The effect of this number of TDL of target cell specificity on the cytotoxic activity of immune lymphocytes was tested in experiments in which mixtures of immune lymphocytes and TDL were tested for cytotoxicity against labelled lymphoma target cells (TABLE 3). No inhibition of cytotoxicity was detected when the TDL comprised up to 40% of the immune cell number. In another experiment in which different

TABLE 4.2

DETACHMENT OF MONOLAYER CELLS DURING ADSORPTION

	LABELLED CELLS (%) [c]	
	EXPT 1. ^{3}H-URIDINE	EXPT 2. ^{3}H-LEUCINE
MONOLAYER CELLS [a]	72.0	92.7
NON-ADHERENT LYMPHOCYTES [b]	2.5	3.2
ADHERENT LYMPHOCYTES [b]	12.7	15.0

a TDL labelled in vitro before preparation of monolayers.

b Unlabelled lymph node cells were incubated on labelled TDL monolayers

c Smears of each cell population were developed for autoradiography. >200 cells counted/slide; >5 grains/cell scored positive.

TABLE 4.3

THE EFFECT OF TDL OF TARGET SPECIFICITY ON THE CYTOTOXIC ACTIVITY OF IMMUNE LYMPHOCYTES

IMMUNE [a] LYMPHOCYTES ($x10^5$)	WF/G_1 TARGET CELLS [b] ($x10^5$)	WF.TDL ($x10^5$)	%SPECIFIC ^{125}I-RELEASE (MEAN ± S.E.)
12.5	0.25	---	30.1 ± 0.8
		5.0	29.0 ± 2.9
		2.5	32.7 ± 1.2
		1.25	28.1 ± 1.4
		0.63	31.2 ± 1.5
		0.32	32.6 ± 0.8
		0.1	28.4 ± 1.8
		0.05	30.4 ± 2.3
		0.025	29.9 ± 2.1

a Lymph node cells from HO rats immunised with WF skin grafts.

b WF/G_1 lymphoma cells labelled with $^{125}IUdR$. 18 hour cytotoxic assay

numbers of immune lymphocytes were incubated with mixtures of labelled tumour cells and unlabelled TDL, no inhibition was detected over a range of lymphocyte/target cell ratios of from 100:1 to 12.5:1 (TABLE 4). These experiments showed that TDL in excess of the estimated numbers of contaminating monolayer cells present in the non-adherent and adherent cell fractions did not affect the cytotoxic activity of these cells under the conditions of assay employed.

Significant enrichment and depletion of cytotoxic activity was obtained after a single adsorption on specific TDL monolayers. Experiments were carried out in which the non-adherent cells were adsorbed three times in succession on fresh TDL monolayers and the cells adherent to each set of monolayers were recovered and tested for cytotoxic activity (FIGURE 2). The non-adherent cells were approximately 50% depleted after a single adsorption and 100% depleted after a second. The cells adherent to the first set of monolayers were greatly enriched in cytotoxic activity; the activity of these cells was increased almost ten-fold compared to the unfractionated cells. The cells adherent in the second adsorption had an activity as great as the unfractionated cells and the cells adherent in the third adsorption were not cytotoxic. Theresults of this and one other experiment (data not shown) showed that most of the cytotoxic cells adhered to the cell monolayers in the first adsorption and that any cytotoxic activity remaining in the non-adherent cell population could be removed by a further incubation on fresh monolayers.

4.2 The specificity of cytotoxic cell adherence.

The adherence of cytotoxic cells to allogeneic cell monolayers was immunologically specific; lymphocytes from HO rats immunised with AO skin grafts were incubated on either AO or DA monolayers and the adherent and non-adherent cells were tested for cytotoxic activity

TABLE 4.4

THE EFFECT OF UNLABELLED TDL OF TARGET CELL SPECIFICITY ON CELL MEDIATED CYTOTOXICITY.

IMMUNE LYMPHOCYTES [a] ($x10^5$)	WF/G_1 TARGET CELLS [b] ($x10^5$)	WF.TDL ($x10^5$)	PER CENT SPECIFIC ^{125}I-RELEASE
25.0	0.25	---	18.4 ± 0.4
25.0	0.25	1.0	19.0 ± 0.8
12.5	0.25	---	10.7 ± 0.7
12.5	0.25	1.0	11.6 ± 0.5
6.25	0.25	---	6.2 ± 1.2
6.25	0.25	1.0	5.1 ± 1.3
3.175	0.25	---	1.8 ± 0.9
3.175	0.25	1.0	1.4 ± 0.6

a Lymph node cells from HO rats immunised with WF skin grafts.
b Labelled with ^{125}IUdR.
c Mean ± S.E. of triplicates after 18 hours incubation.

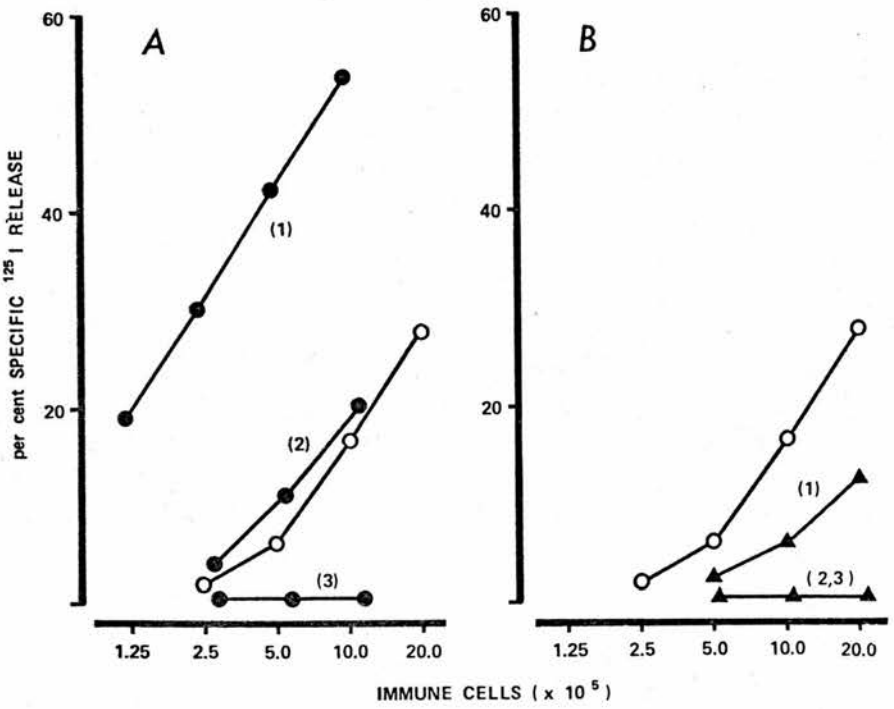

Fig. 4.2 Successive adsorption of cytotoxic lymphocytes on specific allogeneic monolayers.

Non-adherent cells were successively adsorbed three times on fresh TDL monolayers, (Non-adherent 1, 2, 3) and the adherent cells to each set of monolayers were recovered (Adherent 1, 2, 3). Unfractionated cells o——o ; non-adherent cells ▲——▲ ; adherent cells ●——●

against WF/G_1 lymphoma cells (Fig. 3). The relative cytotoxic activity of the cells adherent and non-adherent to specific and third-party allogeneic monolayers was calculated, as before, from the dose response curves obtained in this and one other experiment (TABLE 5). Cells non-adherent to the specific monolayers were substantially depleted of cytotoxic activity and the activity of the adherent cells was significantly increased. In contrast, the activity of the cells non-adherent to the third-party monolayers was not significantly different from the unfractionated cells, and the cells adherent to these monolayers had very little cytotoxic activity.

4.3 Adsorption of lymphocytes on erythrocyte monolayers.

Rat erythrocytes express histocompatibility antigens on their surface and these cells provoke a strong antibody response in an allogeneic recipient (Rolstad 1977). It was of interest, therefore, to compare monolayers of erythrocytes and thoracic-duct lymphocytes as immuno-adsorbents for cytotoxic lymphocytes (Fig. 4). In contrast to the significant enrichment and depletion of cytotoxic activity obtained after fractionation on TDL monolayers, the cells non-adherent to erythrocyte monolayers showed slightly greater activity compared to the unfractionated cells, and the activity of the adherent cells, although significant, was reduced compared to the unfractionated cells. The failure of cytotoxic lymphocytes to adhere to erythrocyte monolayers could be due to a lack of sufficient alloantigens on the surface of these cells. The density and/or spatial representation of specific antigen receptors required to achieve significant binding of cytotoxic lymphocytes *in vitro* may not be the same as that required for the induction of an alloantibody response *in vivo*.

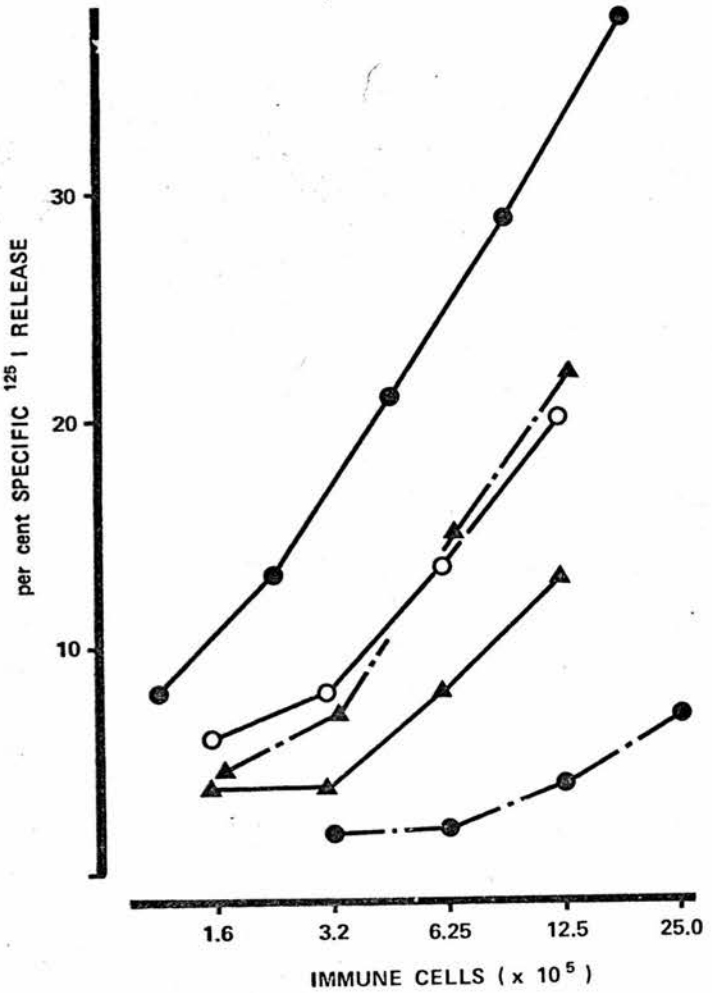

Fig. 4.3 The specificity of cytotoxic cell adherence.

HO anti-Ao lymphocytes were adsorbed on either AO or DA.TDL monolayers and the adherent and non-adherent cells were tested for cytotoxic activity. Unfractionated cells ○———○ ; cells adherent to specific (AO) monolayers ●———● ; cells adherent to third-party (DA) monolayers ●- - - -● ; cells non-adherent to specific monolayefs ▲——▲ ; cells non-adherent to third-party monolayers ▲- -▲ .

TABLE 4.5

THE SPECIFICITY OF CYTOTOXIC CELL ADHERENCE [a]

EXPT.	RELATIVE CYTOTOXIC ACTIVITY [b]			
	NON-ADHERENT CELLS		ADHERENT CELLS	
	SPECIFIC MONOLAYER	THIRD PARTY MONOLAYER	SPECIFIC MONOLAYER	THIRD PARTY MONOLAYER
1	0.56	1.16	1.54	0.14
2	0.42	1.07	1.83	0.04

a Lymph node cells from HO rats immunised with AO skin grafts were incubated on either specific (AO) or third-party (DA) TDL monolayers.

b Compared to unfractionated cells. Relative activity was calculated by a parallel line assay as described in the text. Expt. 2 is illustrated in Figure 3.

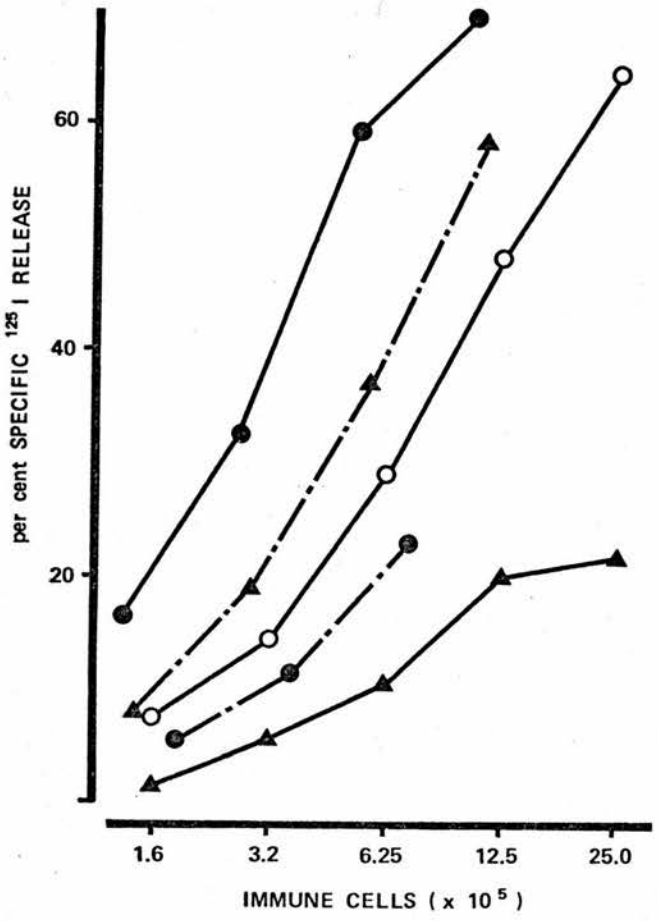

Fig. 4.4 Adsorption of cytotoxic cells on erythrocyte and thoracic-duct lymphocyte monolayers.

DA anti-AO lymphocytes were adsorbed on either AO.TDL or AO.RBC monolayers and the adherent and non-adherent cells were tested for cytotoxic activity. Unfractionated cells ○——○ ; cells adherent to TDL monolayers ●——● ; cells adherent to RBC monolayers ●- - -● ; cells non-adherent to TDL monolayers ▲——▲ ; cells non-adherent to RBC monolayers ▲- - -▲ .

4.4 Adsorption of lymphocytes on monolayers which express all or only some of the immunising alloantigens.

The adherence of cytotoxic lymphocytes to allogeneic cell monolayers is immunologically specific and is presumed to occur via antigen-specific receptors on the surface of the cytotoxic cells which bind to complementary antigenic determinants on the target cell surface. In experiments described in the previous chapter it was found that the cytotoxic response measured after immunisation against AgB antigens alone was at least as great as the response obtained after immunisation against both AgB and non-AgB alloantigens. If the specificity of the majority of the cytotoxic lymphocytes produced after immunisation with a combination of AgB and non-AgB antigens was directed towards the AgB antigens, as these results suggested, then monolayers of cells which express only the immunising AgB alloantigens should be as effective an immuno-adsorbent as monolayers of cells which express the full complement of immunising antigen specificities. Experiments were designed to test this hypothesis. Cytotoxic cell populations were obtained from HO rats immunised with either WF or HO.B2 skin grafts and were incubated on either WF or HO.B2 TDL monolayers. The adherent and non-adherent cells were then tested for cytotoxicity against WF/G_1 lymphoma cells. The results obtained in two separate experiments are illustrated in Figs. 5 and 6. HO anti-WF cells adherent to HO.B2 cell monolayers (which express only the relevant AgB antigens) were as cytotoxic as HO anti-WF cells adherent to WF monolayers which express the full complement of immunising specificities (AgB plus non-AgB). The cytotoxic activity of HO anti-HO.B2 cells adherent to WF monolayers (which express the relevant AgB antigens and irrelevant non-AgB antigens) was slightly reduced compared to the activity of HO anti-HO.B2 cells adherent to homologous cell monolayers. A similar pattern emerged

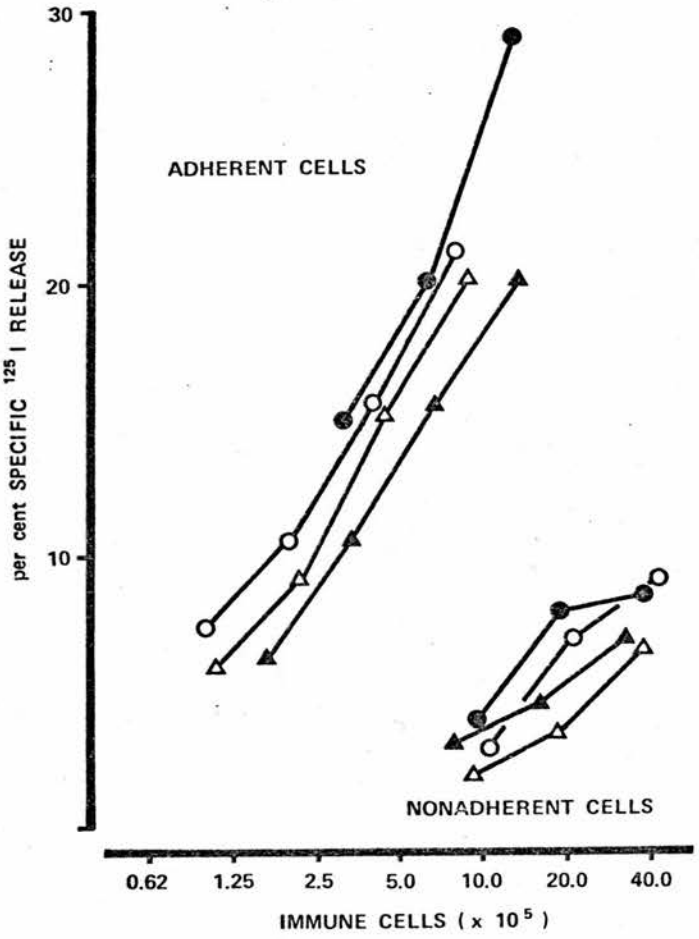

Fig. 4.5 Adsorption of cytotoxic lymphocytes on monolayers which express all or only some of the immunising alloantigens.

Rats were skin grafted in various different strain combinations and the draining lymph node cells obtained eight days later were adsorbed on HO.B2 or WF TDL monolayers. HO anti-WF cells adsorbed on WF monolayers O——O ; HO anti-WF cells adsorbed on HO.B2 monolayers ●——● ; HO anti-HO.B2 cells adsorbed on HO.B2 monolayers △——△ ; HO anti-HO.B2 cells adsorbed on WF monolayers ▲——▲ .

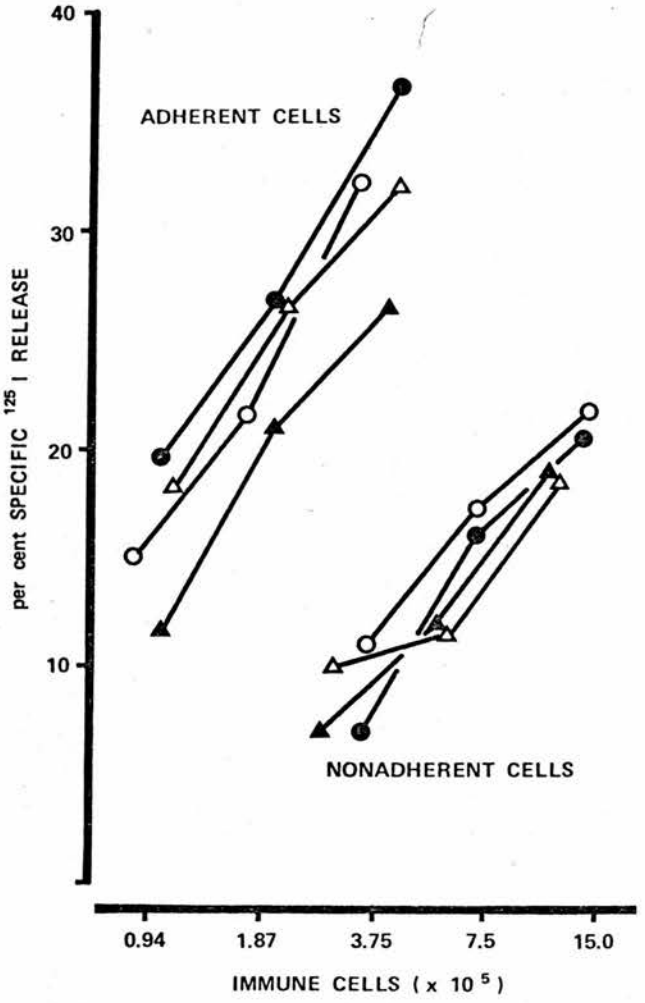

Fig. 4.6 Adsorption of cytotoxic lymphocytes on monolayers which express all or only some of the immunising alloantigens.

Rats were skin grafted in various different strain combinations and the draining lymph node cells obtained eight days later were adsorbed on HO.B2 or WF TDL monolayers. HO anti-WF cells adsorbed on WF monolayers O——O ; HO anti-WF cells adsorbed on HO.B2 monolayers ●——● ; HO anti-HO.B2 cells adsorbed on HO.B2 monolayers △——△ ; HO anti-HO.B2 cells adsorbed on WF monolayers ▲——▲ .

when the cytotoxic activity of the different non-adherent cell populations was compared. HO anti-WF cells were depleted of activity to the same extent after incubation on either WF or HO.B2 monolayers, and the same was true for the HO anti-HO.B2 cells. The results obtained in these experiments substantiate the conclusion drawn in the previous chapter viz. that the majority of the cytotoxic lymphocytes produced after immunisation with a combination of AgB and non-AgB antigens have a specificity directed towards the AgB alloantigens.

4.5 Inhibition of cell-mediated cytotoxicity (CMC) by unlabelled target cells.

The failure of TDL of target specificity to inhibit cell-mediated cytotoxicity (see 4.1 above) cannot be attributed to the absence of antigen receptors for the cytotoxic lymphocytes on these cells, since cytotoxic cells adhere to TDL monolayers and the adherence is immunologically specific (4.4 above). The maximum ratio of unlabelled TDL to labelled lymphoma target cells used in the experiments described in section 4.1 was 20:1, and it has been reported that successful inhibition of CMC required a fifty-fold excess of unlabelled target cells; five to twenty-fold excesses resulted in only marginal inhibition after a short incubation period (Kedar and Bonavida 1975). Cytotoxic lymphocytes can also kill more than one target cell and the incubation time of the assay will therefore affect the degree of inhibition observed. It may be that the 18 hour incubation time used in these experiments was too long to observe the effects of target cell competition.

In contrast to the failure of significant numbers of normal thoracic-duct lymphocytes of target cell specificity to inhibit CMC, unlabelled tumour cells were found to be highly effective. In experiments in which mixtures of immune lymphocytes and unlabelled

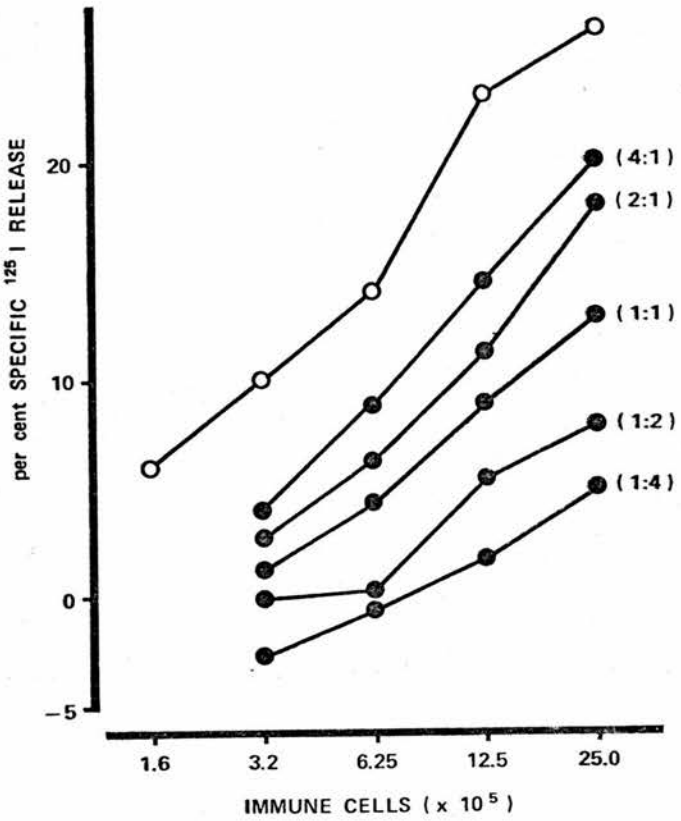

Fig. 4.7. Inhibition of cell-mediated cytotoxicity by unlabelled tumour target cells.

Cell mixtures consisted of different numbers of lymphocytes from HO rats immunised with WF skin grafts, 2.5 x 10^4 ^{125}I-labelled WF/G_1 tumour cells and different numbers of unlabelled WF/G_1 tumour cells. Cytotoxicity was measured after 18 hours. The ratio of labelled to unlabelled tumour cells is given in parentheses. Immune lymphocytes and labelled tumour cells O——O ; mixtures of immune lymphocytes, labelled tumour cells and unlabelled tumour cells ●——● .

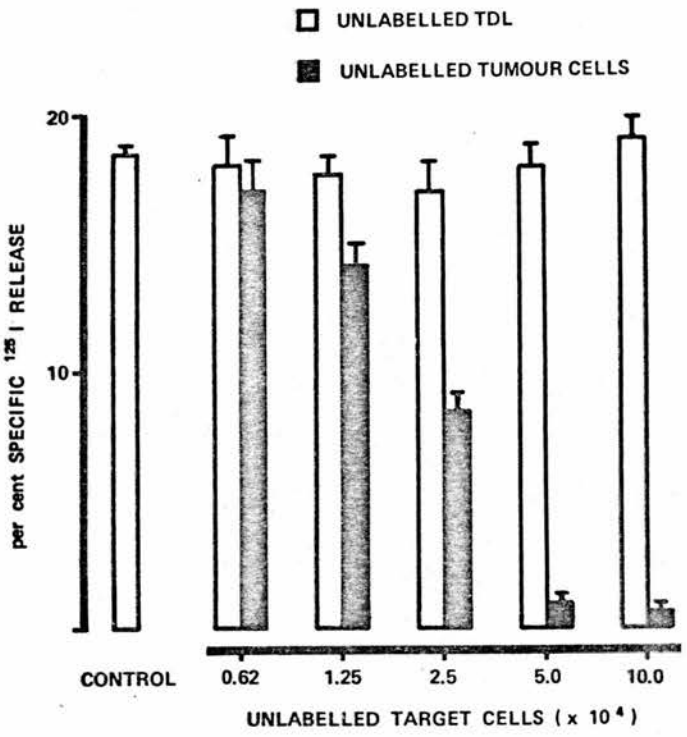

Fig. 4.8 Inhibition of cytotoxicity by unlabelled target cells.

The bars represent mean per cent specific ^{125}I-release ± S.E. of triplicates after 18 hours. Cell mixtures consisted of 25.0 x 10^5 lymph node cells from HO rats immunised with WF skin grafts, 2.5 x 10^4 ^{125}I-labelled WF/G_1 lymphoma cells and different numbers of unlabelled WF/G_1 lymphoma cells (■) or WF.TDL (□).

tumour target cells were tested for cytotoxicity against labelled tumour cells, significant inhibition was detected over a wide range of lymphocyte/labelled target cell ratios and was proportional to the number of unlabelled tumour cells (Fig. 7). The results of another experiment, which directly compared the cytotoxic activity of mixtures of immune lymphocytes, labelled tumour cells and unlabelled tumour cells or normal TDL, are given in Fig. 8. Cytotoxicity was completely inhibited in mixtures which contained a four-fold excess of unlabelled WF/G_1 tumour cells: no inhibition was observed in mixtures containing the same number of unlabelled WF TDL. The failure of TDL to inhibit CMC under conditions in which tumour cells completely abrogate the response is interesting. Thoracic-duct lymphocytes are considerably smaller than the WF/G_1 lymphoma cells and it is possible that the tumour cells can compete more effectively because of their greater size. Support for this interpretation is found in similar experiments reported by Kedar and Bonavida (1975) who showed that in a tumour allograft system cytotoxicity was inhibited more effectively by either tumour cells or Con-A induced blast cells than by normal spleen cells. Alternatively it is possible that the WF/G_1 tumour cells express more histocompatibility antigens on their surface than do thoracic-duct lymphocytes, and it may be that the same is true for mitogen-transformed lymphocytes.

DISCUSSION.

Thoracic-duct lymphocyte monolayers were found to be effective immuno-adsorbents for the cytotoxic lymphocytes produced in response to allogeneic skin grafts: lymphoid cell populations non-adherent to monolayers of target cell specificity were substantially depleted of

cytotoxic activity, and the adherent cells eluted from the monolayers with EDTA were substantially enriched in activity. The adherence of cytotoxic lymphocytes to TDL monolayers was immunologically specific; cells non-adherent to monolayers of alloantigen specificity unrelated to the antigens of the skin graft were not depleted of cytotoxic lymphocytes, and the cells adherent to these monolayers had little or no cytotoxic activity. A quantitative assessment of the extent of depletion of and enrichment in cytotoxic activity was obtained by routinely measuring the activity of the separated cell populations over a range of lymphocyte/target cell ratios and comparing the numbers of cells required to give the same cytotoxic response. This comparison was justified by the fact that, at intermediate ratios, a plot of percentage ^{125}I-release against the logarithm of immune cell number yielded a straight line, the slope of which was the same for the different cell populations within any one experiment. The cells non-adherent to specific allogeneic monolayers were 50-70% reduced in cytotoxic activity after a single adsorption, and this reduction was accompanied by a two to five-fold enrichment in activity in the adherent cell populations. The cytotoxic lymphocytes remaining in the non-adherent cell populations after a single adsorption were removed by a second adsorption on fresh monolayers.

The degree of specific enrichment in cytotoxic activity in the adherent cell populations obtained in these experiments has not been reported elsewhere. Cytotoxic cells have been recovered from fibroblast monolayers by trypsinisation (Golstein et al. 1971, Berke and Levey 1972) but these workers failed to obtain cell populations substantially enriched in cytotoxic lymphocytes. This may have been in part due to the high proportion (50%) of irrelevant cells which adhered to the

monolayers (Golstein et al. 1971) and also to the inhibitory action of trypsin on the cytotoxic cells, the effects of which persist for several hours after removal of the enzyme (Mauel et al. 1970). EDTA does not inhibit cell-mediated cytotoxicity at the concentration required to elute the cytotoxic cells from the monolayers (Stulting and Berke 1973); and experiments presented in the following chapter, in which lymphocytes were labelled with radioactive uridine prior to adsorption on either homologous or heterologous cell monolayers, suggested that, in contrast to the system used by Golstein et al., the proportion of lymphocytes which adhered non-specifically to TDL monolayers was not significantly greater than the proportion of cells which adhered specifically (Table 3, Ch. 5.3). Substantial binding of cytotoxic cells to fibroblast or macrophage monolayers requires prolonged incubation periods (2-4 hours), whereas adsorption on TDL monolayers occurs within 15-30 minutes, using the adsorption/centrifugation technique described by Kedar et al. (1974b). The amount and distribution of histocompatibility antigens on different cell types is not the same (Aoki et al. 1969) and lymphocytes may have a greater surface density of the appropriate antigens than do fibroblasts. It may be, therefore, that the use of lymphocyte monolayers, together with the short term incubation/centrifugation technique, provided conditions which not only facilitated the antigen specific adherence of cytotoxic lymphocytes, but which also reduced the level of non-specific cell adherence.

The adherence of cytotoxic lymphocytes to monolayers of target cells exploits the fact thatthe cytotoxic reaction involves direct contact between the effector and target cells, which is presumed to be mediated by antigen-specific receptors on the surface of the cytotoxic cells which bind to complementary antigenic determinants on the target

cell surface. Monolayer adsorption studies provide indirect evidence for the existence of suchreceptors and have been used to examine the heterogeneity of cytotoxic lymphocytes in cell populations obtained after immunisation with more than one alloantigen specificity. Golstein et al. (1971) showed that,in mice immunised with two different allogeneic tumours,there were two distinct populations of cytotoxic lymphocytes,each of which carried only one type of receptors; and in similar adsorption experiments Brondz and Snegirova (1971) demonstrated that differences for both H-2^k and H-2^d loci between donors and recipients of tumour cells led to the formation of two populations of cytotoxic cells, each reacting with only one of the two sets of antigens. In the experiments reported here, a comparison of the relative enrichment and depletion of cytotoxic cells,after incubation on cell monolayers expressing all or only some of the immunising antigens,showed that the majority of the cytotoxic cells produced after immunisation with a combination of AgB and non-AgB antigens had a specificity directed towards the AgB antigens. These results are in agreement with the recent findings of Pevey and Pierce (1975) who showed that spleen cells obtained from mice immunised with H-2 and non-H-2 antigens were depleted of cytotoxic cells to the same extent after incubation on cell monolayers which expressed either the full complement of immunising specificites, or only the H-2 antigens.

CHAPTER FIVE

THE ADHERENCE OF GvH-REACTIVE CELLS TO ALLOGENEIC CELL MONOLAYERS.

The responses of thymus-derived (T) lymphocytes to alloantigens determined by the major histocompatibility complex (MHC) have been demonstrated by several different methods, including graft-versus-host (GvH) reactions (Simonsen 1962a), proliferation in mixed lymphocyte culture (MLC) (Dutton 1965, Wilson 1967) and the generation of cytotoxic effector cells (Brunner et al. 1968). It has not yet been clearly established whether these different methods of measuring cell-mediated immunity are functions of distinct T-cell subpopulations, or whether they reflect the different activities of a single T cell lineage.

One approach to this problem is to positively select or to deplete cell populations on the basis of one function, and to assess which other functions are co-selected or depleted.

GvH and MLC responses are measures of cell proliferation and can be used to measure the capacity of virgin antigen-reactive cells to respond to alloantigens. Immunisation against a major histocompatibility antigen produces little or no change in GvH activity (Simonsen 1962, Ford and Simonsen 1971). In contrast, immunisation is a prerequisite for cytotoxic effector cell production. The fact that immunisation produced cytotoxic lymphocytes with apparently little or no change in the number of GvH-reactive cells suggested the possibility that, in immune cell populations, cytotoxic lymphocytes and GvH-reactive cells belonged to distinct cell populations. If this is the case, then it should be possible to separate the two cell populations; selection for or against one function without selection for or against the other would be strong evidence for the non-identity of the cells involved. The experiments presented in the previous chapter showed that cell populations could be obtained which were either depleted of or enriched in cytotoxic lymphocytes by incubation <u>in vitro</u> on specific allogeneic

monolayers. The experiments described in this chapter investigated the effects of such an enrichment and depletion on the GvH activity of the same cell populations.

RESULTS

5.1 The GvH activity of lymph node cell populations enriched in and depleted of cytotoxic lymphocytes.

Lymphocytes from HO rats immunised with AO skin grafts were incubated on AO.TDL monolayers and the adherent and non-adherent cells were tested for both cytotoxic and GvH activity. The results of a representative experiment are presented in Fig. 5.1. As expected, the cytotoxic activity of the adherent cells was significantly increased and the activity of the non-adherent cells was substantially reduced compared to the unfractionated cells. In contrast, the GvH activity of both the non-adherent and the adherent cells was the same as the activity of the unfractionated cells. The dose response curves for the different cell populations were parallel,or very nearly so,in both assays and this was a consistent finding in different experiments. The GvH and cytotoxic activity of the adherent and non-adherent cells compared to the unfractionated cells could therefore be calculated,as before,from the horizontal distance between the dose response curves obtained in each assay. The results of four separate experiments calculated in this way, including the experiment illustrated in Fig.1, are summarised in TABLE 1. There was a consistent and substantial depletion of and enrichment in cytotoxic activity in the non-adherent and adherent cell populations respectively,in every experiment. In contrast, there was no difference in the GvH activity of the non-adherent and the unfractionated cells, and in only one experiment was the activity of the adherent cells significantly increased compared to the unfractionated

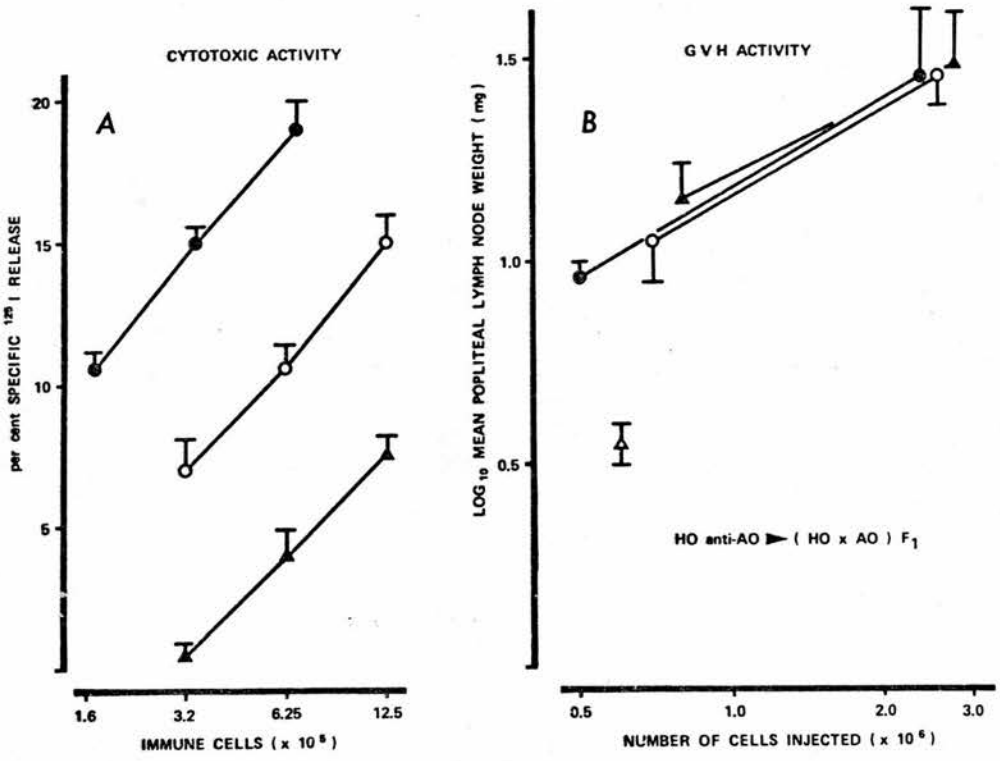

Fig. 5.1 The cytotoxic activity and GvH activity of lymphocytes adherent and non-adherent to allogeneic cell monolayers.

Lymph node cells from HO rats immunised with AO skin grafts were incubated on AO.TDL monolayers. The adherent and non-adherent cells were tested both for cytotoxic activity (A) and GvH activity (B) against the immunising AgB antigens. Unfractionated cells ○——○ ; non-adherent cells ▲——▲ adherent cells ●——● .

TABLE 5.1

THE CYTOTOXIC AND GvH ACTIVITY OF LYMPHOCYTES ADHERENT AND NON-ADHERENT TO ALLOGENEIC CELL MONOLAYERS.[a]

EXPT.	CYTOTOXIC ACTIVITY [b]		GvH ACTIVITY [b]	
	NON-ADHERENT CELLS	ADHERENT CELLS	NON-ADHERENT CELLS	ADHERENT CELLS
1.	0.0	2.98	1.1 ($p > 0.25$) [c]	0.7 ($p > 0.25$)
2.	0.48	2.94	1.1 ($p > 0.25$)	0.87 ($p > 0.25$)
3.	0.20	3.17	1.32 ($p > 0.25$)	1.06 ($p > 0.25$)
4.	0.42	3.01	0.74 ($p > 0.25$)	2.24 ($p < 0.005$)
Mean	0.28 ± 0.10	3.03 ± 0.05	1.07 ± 0.12	1.22 ± 0.35

a Lymph node cells from HO rats immunised with AO skin grafts incubated on AO.TDL monolayers.

b Compared to unfractionated cells as described in the text. Expt. 3 is illustrated in Fig. 1.

c Statistical comparison of the GvH activity of the fractionated cells compared to the unfractionated cells was by analysis of co-variance. In no experiment was there a significant difference in the slope of the regression line for the different cell populations ($p > 0.10$).

cells (Expt. 4). When the results of all four experiments were averaged, the GvH activity of both the non-adherent and adherent cells was not significantly different from the unfractionated cells; the mean potency ratios (which compare the numbers of cells in each population required to produce the same lymph node enlargement as the unfractionated cells) were 1.07 and 1.22 respectively for the non-adherent and adherent cell populations. The GvH activity of each cell population was very similar in different experiments and, when the results of the four experiments summarised in Table 1 were plotted on the same graph, the regression lines for the adherent and non-adherent cell populations, calculated by the method of least squares, were coincident and not significantly different from the regression line for the unfractionated cells (Fig. 2).

A small proportion of the monolayer cells become detached during the adsorption procedure and these cells are present in different proportions in the adherent and non-adherent cell populations (see Ch. 4.1). In the experiments described above, both the monolayer cells (AO) and the immune cells (HO) would be capable of eliciting a GvH reaction in the (AO x HO)F_1 hybrid. Although the number of monolayer cells which become detached is small, the presence of different numbers of these cells in the adherent and non-adherent cell populations might affect the GvH activity of these cells to a different extent. The results of an experiment in which the monolayer cells were syngeneic to the F_1 hybrid and could not therefore themselves contribute to the GvH activity of the immune cell populations are presented in Fig. 3. The results were identical to those obtained in the previous experiments; selection for and against cytotoxic activity was not accompanied by selection for or against GvH activity.

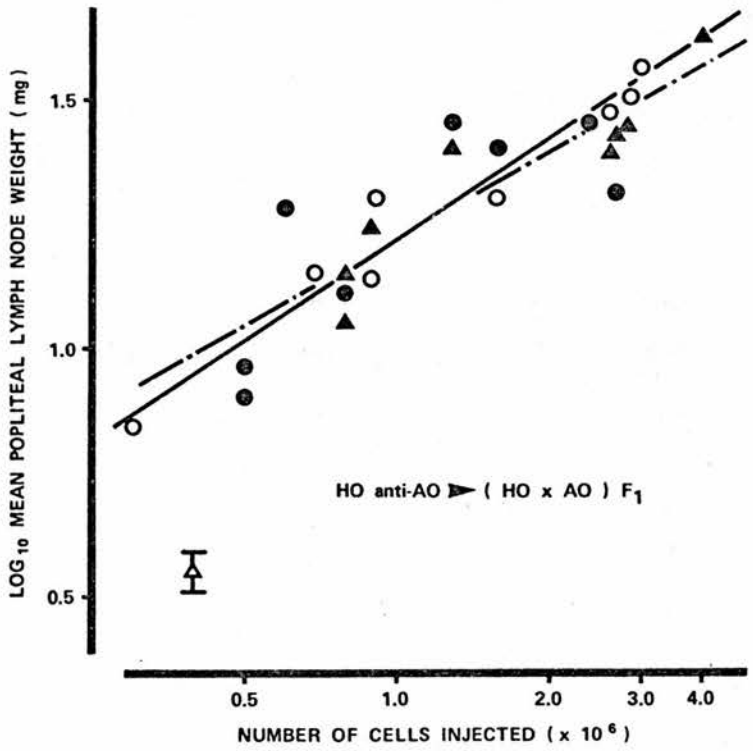

Fig. 5.2. The GvH activity of lymphocytes adherent and non-adherent to allogeneic cell monolayers.

Lymph node cells from HO rats immunised with AO skin grafts were incubated on AO.TDL monolayers and the adherent and non-adherent cells were tested for GvH activity against the immunising antigens. Unfractionated cells O——O ; non-adherent cells ▲- - -▲ ; adherent cells ●— - —● ; The data from four separate experiments (see Table 5.1) were plotted on the same graph. Each point represents the mean weight of four lymph nodes. The regression lines for the adherent and non-adherent cells were coincident and not significantly different from the regression line for the unfractionated cells.

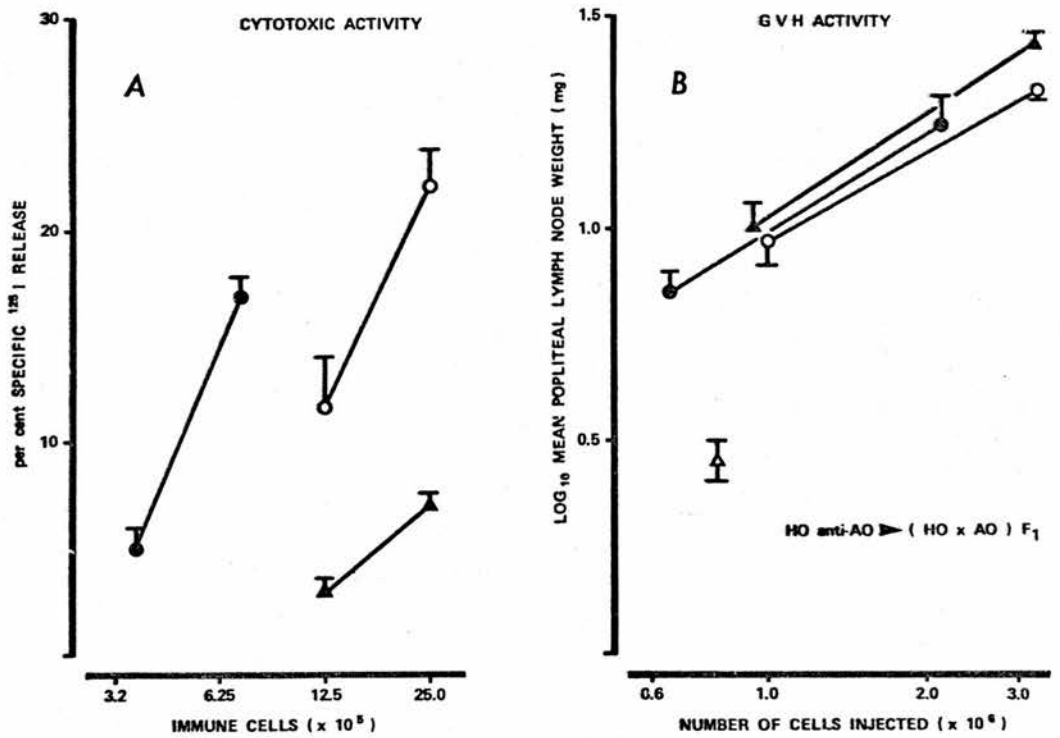

Fig. 5.3. The cytotoxic activity and GvH activity of lymphocytes adherent and non-adherent to semi-allogeneic cell monolayers.

Lymph node cells from HO rats immunised with AO skin grafts were incubated on (HO × AO).TDL monolayers and tested for cytotoxic activity (A) and GvH activity (B) against the immunising antigens. Unfractionated cells O——O ; non-adherent cells ▲——▲ ; adherent cells ●——●.

5.2 The Specificity of GvH-reactive cell Adherence.

The experiments presented in the previous section failed to detect significant antigen specific enrichment of GvH-reactive cells in lymphoid cell populations adherent to allogeneic cell monolayers. However, the adherent cell populations had at least as great GvH activity as the starting population. This GvH activity may have been attributable either to the cytotoxic lymphocytes themselves, or to a distinct population of GvH-reactive cells which also adhered to the monolayers. While the failure to detect a reduction in the GvH activity of the non-adherent cell populations made it unlikely that significant numbers of GvH-reactive cells adhered specifically to the monolayers, it remained possible that small numbers of cells did so, or, alternatively, that GvH-reactive cells adhered to the monolayers in an immunologically non-specific way i.e. for reasons other than their antigen specific receptors. With these possibilities in mind, experiments were designed to examine the specificity of GvH-reactive cell adherence to allogeneic cell monolayers, using both non-immune cell populations which contain GvH-reactive cells, but which are devoid of cytotoxic lymphocytes and immune cell populations which have both cytotoxic and GvH activity.

The results of an experiment in which non-immune HO lymph node cells were incubated on either AO or HO.TDL monolayers and the adherent cells were tested for GvH activity in (HO x AO)F_1 hybrids are presented in Fig. 4. This experiment failed to demonstrate any antigen specific enrichment of GvH-reactive cells in the lymphocytes adherent to allogeneic cell monolayers; the GvH activity of these cells was in fact slightly reduced compared to the unfractionated cells, although the difference was not statistically significant. Although GvH-reactive cells with specificity for AO alloantigens adhered to AO monolayers, there was no evidence that these cells were present to a significantly

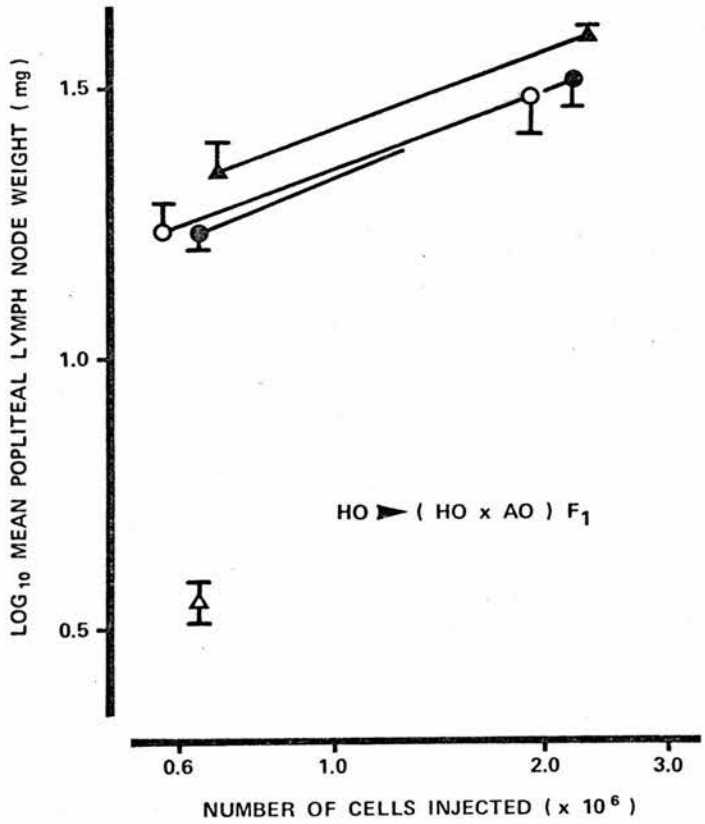

Fig. 5.4. The GvH activity of non-immune lymphocytes adherent to allogeneic and syngeneic cell monolayers.

Lymph node cells from non-immunised HO rats were incubated on either syngeneic (HO) or allogeneic (AO) TDL monolayers and the adherent cells were tested for GvH activity in $(HO \times AO)F_1$ hybrids. Unfractionated cells ▲——▲ ; cells adherent to allogeneic monolayers ●——● ; cells adherent to syngeneic monolayers ○——○ . There was no significant difference in the activity of the three cell populations ($p > 0.05$)

greater extent in the cells adherent to the allogeneic monolayers than in the cells adherent to syngeneic monolayers; the GvH activity of both cell populations against the (HO x AO)F_1 hybrid was the same. The adherence of lymphocytes to syngeneic cell monolayers cannot be a consequence of alloantigen recognition and these results suggested, therefore, that the adherence GvH-reactive cells to monolayers was immunologically non-specific. Similar results were obtained in experiments in which lymph node cells from AO rats were incubated on HO monolayers and the non-adherent and adherent cells were tested for GvH activity in both specific (AO x HO) and third-party (AO x DA)F_1 hybrids. The results obtained in three separate experiments were plotted on the same graph (Fig. 5). GvH-reactive cells with specificity for the monolayer (HO) alloantigens did not represent a greater proportion of the adherent cell population than did GvH-reactive cells with specificity for unrelated (DA) alloantigens; the adherent cells were neither enriched in activity against the (AO x HO)F_1 hybrid, nor depleted of activity against the (AO x DA)F_1 hybrid.

These experiments established that GvH-reactive cells present in non-immune cell populations adhered non-specifically to allogeneic monolayers under these conditions of adsorption. The specificity of adherence of GvH-reactive cells in immune cell populations was investigated in a similar series of experiments. Lymph node cells from HO rats immunised with AO skin grafts were incubated on either specific allogeneic (AO) or syngeneic (HO) monolayers and the adherent cells were tested for GvH activity in (HO x AO)F_1 hybrids. The results of two separate experiments plotted on the same graph are presented in Fig. 6. The results were identical to those obtained in previous experiments in that there was no evidence of enrichment in GvH activity in the cells adherent to the allogeneic monolayers. These experiments

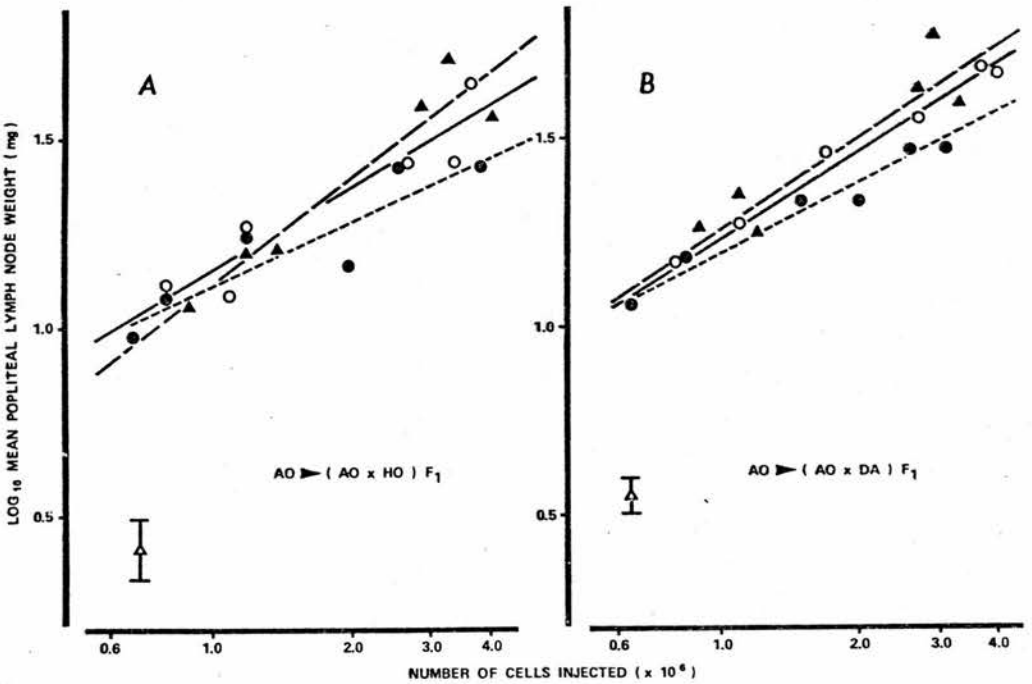

Fig. 5.5 The lack of specificity of non-immune GvH-reactive cell adherence.

Lymph node cells from non-immunised AO rats were incubated on HO.TDL monolayers and the adherent and non-adherent cells were tested for GvH activity against the monolayer alloantigens (A) and against third-party alloantigens (B). Unfractionated cells O———O ; non-adherent cells Δ— ——Δ; adherent cells ●- - - - -● . There was no significant difference in the GvH activity of the different cell populations in either the (AO x HO)F_1 or (AO x DA)F_1 hybrids ($p > 0.05$).

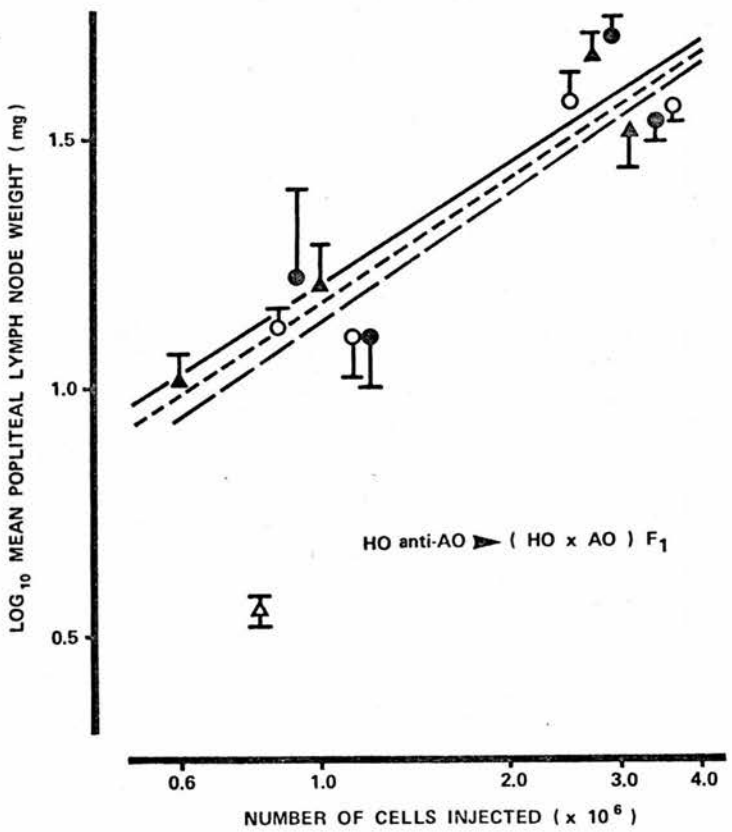

Fig. 5.6. The GvH activity of immune lymphocytes adherent to allogeneic and syngeneic cell monolayers.

Lymph node cells from HO rats immunised with AO skin grafts were incubated on either specific allogeneic (AO) or syngeneic TDL monolayers and the adherent cells were tested for GvH activity against the immunising antigens. Unfractionated cells ▲——▲ ; cells adherent to specific allogeneic monolayers ●- - - -● ; cells adherent to syngeneic monolayers ○— —○ . There was no significant difference in the activity of the three cell populations ($p > 0.05$).

also showed that GvH-reactive cells in immune cell populations adhered to the same extent to both allogeneic and syngeneic cell monolayers; the activity of the two adherent cell populations was the same.

Immune cell populations adherent to specific allogeneic monolayers are enriched in cytotoxic lymphocytes (Fig. 1, Table 1) and very few cytotoxic cells adhere to third-party TDL monolayers (Ch. 4, Table 5). Therefore, the population adherent to syngeneic cell monolayers would be expected to contain very few cytotoxic cells, whereas the cells adherent to the allogeneic monolayers would be expected to be enriched in cytotoxic lymphocytes. The demonstration that these cell populations had the same GvH activity (Fig. 6) suggested that the cytotoxic lymphocytes did not make a substantial contribution to the GvH response, although it should be pointed out that cytotoxic data was not obtained in these particular experiments.

In a second series of experiments, lymph node cells from HO rats immunised with AO skin grafts were incubated on AO monolayers and the non-adherent and adherent cells were tested for GvH activity in both specific (HO x AO) and third-party (HO x DA)F_1 hybrids. The results of two separate experiments were plotted on the same graph (Fig.7). There was some evidence for antigen specific enrichment in GvH activity in the immune cells adherent to allogeneic monolayers, in that the activity of these cells against the specific (HO x AO)F_1 hybrid was slightly increased compared to the unfractionated cells. The difference was not statistically significant, however, and was not accompanied by a reduction in the activity of the non-adherent cells in the same F_1 hybrid. The GvH activity of the adherent cells against the third-party (HO x DA)F_1 hybrid was, however, significantly reduced. This finding was in contrast to the results obtained in similar experiments using non-immune cell populations in which it was found that HO GvH-reactive

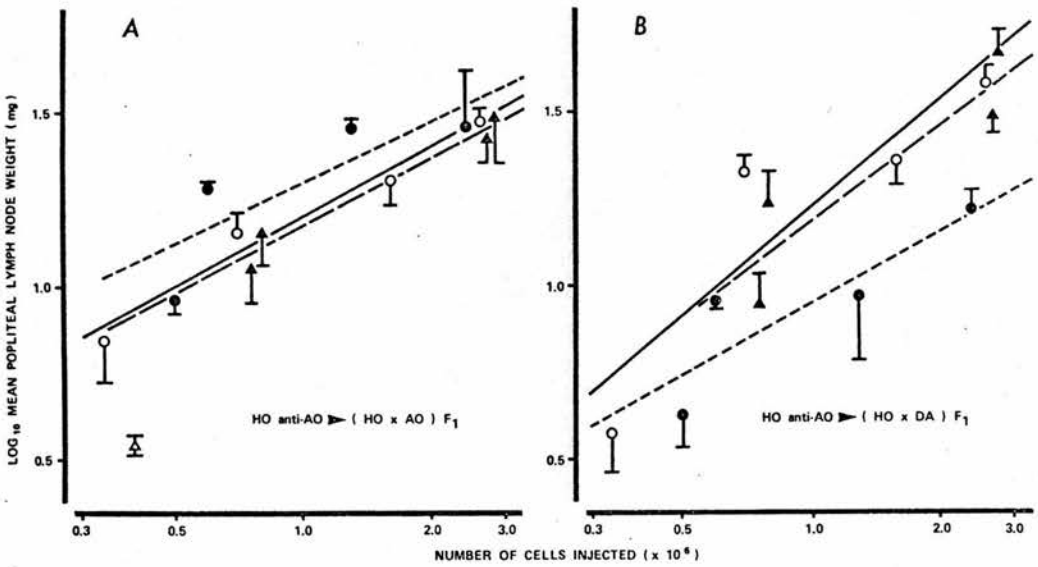

Fig. 5.7 The lack of specificity of immune GvH-reactive cell adherence.

Lymph node cells from HO rats immunised with AO skin grafts were incubated on specific allogeneic monolayers and were tested for GvH activity against the immunising antigens (A) and third-party antigens (B). Unfractionated cells O——O ; non-adherent cells ▲– – –▲ ; adherent cells ●- - - -●. There was no difference in the activity of the different cell populations in the (HO x AO)F_1 hybrids ($p > 0.05$). The adherent cells were significantly reduced in activity in the (HO x DA)F_1 hybrids ($p < 0.01$).

cells with specificity for DA alloantigens adhered to AO monolayers to the same extent as did cells with specificity for the monolayer alloantigens (Fig. 5). The immune cell populations adherent to the monolayers in these experiments would, however, be enriched in cytotoxic lymphocytes with specificity for AO alloantigens and these cells would not be expected to participate in a GvH reaction against DA alloantigens. The reduction in the activity of the adherent cells against the (HO x DA)F_1 hybrid is likely, therefore, to have been the result of dilution of the GvH reactive cells with specificity for DA alloantigens (which adhered non-specifically to the monolayers) by the adherent cytotoxic lymphocytes with specificity for AO alloantigens.

In summary, there was no evidence for antigen specific binding of GvH-reactive cells to allogeneic TDL monolayers under these conditions of adsorption. GvH-reactive cells adhered to the monolayers, but the cells with specificity for the monolayer antigens were neither removed from the non-adherent cell populations nor were they present in the adherent cell populations to any greater extent than were GvH-reactive cells with specificity for unrelated alloantigens.

5.3 The proportion of Lymphocytes which adhered to allogeneic cell monolayers.

The functional studies described so far showed clearly that cytotoxic lymphocytes adhered specifically to allogeneic cell monolayers under conditions in which GvH-reactive cells did not. The experiments presented here were designed to investigate the possibility that antigen specific cell adherence could also be measured on the basis of a difference in the numbers of immune lymphocytes which adhered to homologous and heterologous cell monolayers. By using different radioactive precursors, viz. uridine and thymidine, to label lymphocytes,

the adherence of different lymphocyte sub-populations was measured.

The basic experimental design is illustrated in Fig. 8. In the first series of experiments, lymph node cells from HO rats immunised with either AO or DA skin grafts were labelled separately in vitro with either ^{3}H- or ^{14}C-uridine and mixtures of the cell populations were prepared as shown in FIG. 8. The cell mixtures were then incubated on AO.TDL monolayers, the non-adherent and adherent cells were recovered by standard procedures, and aliquots of each cell population were counted for ^{3}H and ^{14}C. After removal of the adherent cells eluted from the monolayers by EDTA, the monolayers were digested and counted for ^{3}H and ^{14}C so that any adherent cells still attached to the monolayers could be included in the estimates of total radioisotope adherent to the monolayers. Because lymphocytes labelled in vitro with ^{3}H-uridine lose ^{3}H faster than cells labelled with ^{14}C-uridine lose ^{14}C (Ford and Simmonds 1972), the differential elution of isotope from the cells during the adsorption procedure was fully compensated for in each experiment by preparing and fractionating duplicate mixtures of the two immune cell populations with exchange of the ^{3}H and ^{14}C-uridine (Mixtures A and B, Fig. 8).

A "balance sheet" of the amounts of ^{3}H and ^{14}C present in the different cell fractions of each mixture in a representative experiment is presented in TABLE 2. If significantly more HO anti-AO cells adhered to AO monolayers than did HO anti-DA cells, then this should be reflected in an increase of ^{3}H over ^{14}C in the adherent fraction of Mixture A, and an increase of ^{14}C over ^{3}H in the adherent fraction of mixture B. This was found to be the case; the $^{3}H/^{14}C$ ratio was increased compared to the starting population in the adherent cells in mixture A, and was reduced in the adherent cells in mixture B. In neither mixture was the $^{3}H/^{14}C$ ratio in the non-adherent cell populations

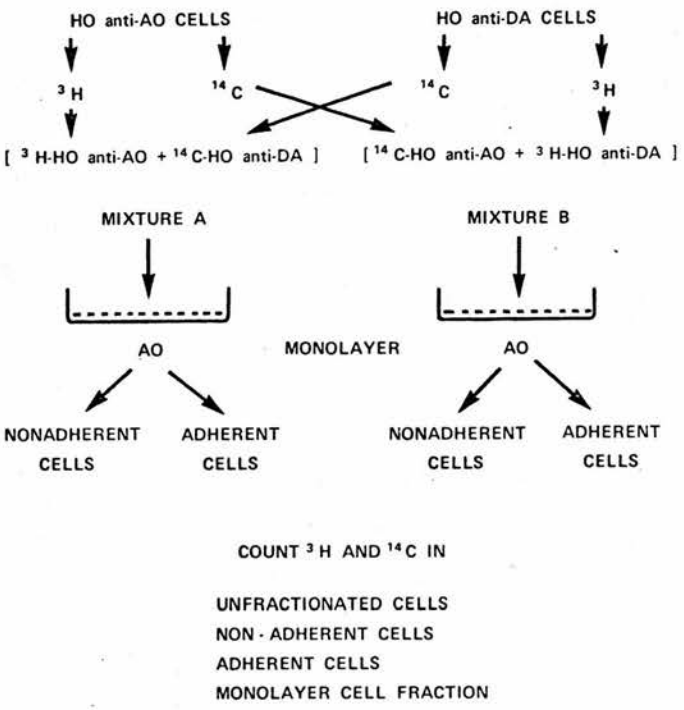

Fig. 5.8 Experimental design used to estimate the proportions of lymphocytes which adhered to homologous and heterologous cell monolayers.

Mixtures of different immune cell populations, labelled separately with ^{3}H and ^{14}C, were incubated on allogeneic TDL monolayers. Differences in the proportions of HO anti-AO and HO anti-DA cells which adhered to AO monolayers were indicated by changes in the ratio of the two isotopes in the non-adherent and adherent cell populations.

TABLE 5.2

MONOLAYER ADHERENCE OF URIDINE LABELLED IMMUNE CELL POPULATIONS [a]

	MIXTURE A ^{3}H-labelled HO anti-AO cells ^{14}C-labelled HO anti-DA cells			MIXTURE B ^{3}H-labelled HO anti-DA cells ^{14}C-labelled HO anti-AO cells		
	^{3}H dpm	^{14}C dpm	$^{3}H/^{14}C$	^{3}H dpm	^{14}C dpm	$^{3}H/^{14}C$
UNFRACTIONATED CELLS			8.24			9.3
NON-ADHERENT CELLS (A)	1,345,950	153,150	8.8	1,970,080	180,180	10.9
ADHERENT CELLS (B) (EDTA-eluted)	125,240	8,280	15.1	53,630	9,570	5.6
ADHERENT CELLS (C) (Retained on monolayers) [b]	66,438	5,839	12.0	63,139	17,210	8.1
$\frac{\text{TOTAL ADHERENT}}{\text{TOTAL RECOVERED}} \times 100$ [c]	12.4	8.4		5.6	12.9	

a Lymph node cells from HO rats immunised with AO or DA skin grafts 8 days previously were incubated on AO monolayers.

b After removal of the adherent cells eluted with EDTA, the monolayers were digested *in situ* and counted for ^{3}H and ^{14}C.

c $\frac{B + C}{A+B+C} \times 100$

TABLE 5.3

URIDINE-LABELLED IMMUNE CELL ADHERENCE [a]

EXPT.	%HO ANTI-AO ACTIVITY ADHERENT TO AO MONOLAYERS (A) [b]	%ANTI-DA ACTIVITY ADHERENT TO AO MONOLAYERS (B)	SPECIFIC ADHERENCE INDEX. $\frac{A}{B}$ [c]
1.	12.7	7.0	1.81
2.	8.5	5.1	1.66
3.	11.5	8.6	1.33
4.	10.4	6.0	1.73
Mean $\pm$ S.E.	10.8 $\pm$ 0.9	6.7 $\pm$ 0.7	1.63 $\pm$ 0.1

a Mixtures of HO anti-AO and HO anti-DA cells labelled with ^{3}H- and ^{14}C-uridine as illustrated in Fig. 5.8 were incubated on AO.TDL monolayers.

b Per cent adherence was calculated as described in the text.

c A ratio of 1.0 indicates no preferential adherence of immune cells to specific monolayers; ratios of greater than unity indicate that more HO anti-AO cells adhered to AO monolayers than did HO anti-DA cells.

significantly altered. When the results were expressed as a percentage of the total isotope recovered, the amount of ^{3}H in the adherent fraction of mixture A was greater than the amount of ^{14}C and the converse was true for mixture B (Table 2, line 5). Taking the mean of the results obtained for the two mixtures, 12.7% of the label in the HO anti-AO cells and 7.0% of the label in the HO anti-DA cells was recovered in the adherent cell fractions. The results of this and three other identical experiments are summarised in Table 3. In each experiment the percentage of the activity in the HO anti-AO cells which was recovered in the adherent cell fractions was significantly greater than the percentage of the activity in the HO anti-DA cells recovered in the same cell fractions and the difference was consistent in different experiments.

Whereas both large and small lymphocytes are labelled in vitro with ^{3}H- and ^{14}C-uridine, only large lymphocytes which are actively synthesising DNA are labelled in vitro with radioactive thymidine. The peak cell proliferation in the lymph nodes draining the site of skin allografts in the rat occurs 6-8 days after grafting, as assessed by ^{3}H-thymidine labelling in vivo (Tilney and Ford 1974). It was considered, therefore, that in vitro thymidine labelling of the lymphocytes obtained from the draining lymph nodes eight days after skin grafting would restrict the lymphocyte population labelled to those cells which had responded to the graft, at least to a greater extent than would labelling with uridine, and that this might therefore be a more sensitive means of detecting antigen specific adherence to allogeneic monolayers.

The results of a series of experiments which were identical to the preceding except that the immune cells were labelled with ^{3}H- and ^{14}C-thymidine instead of uridine, are presented in Tables 4 and 5. As before,

TABLE 5.4

MONOLAYER ADHERENCE OF THYMIDINE-LABELLED IMMUNE CELL POPULATIONS [a]

	MIXTURE A ^{3}H-labelled HO anti-AO cells [a] ^{14}C-labelled HO anti-DA cells			MIXTURE B ^{3}H-labelled HO anti-DA cells ^{14}C-labelled HO anti-AO cells		
	^{3}H dpm	^{14}C dpm	$^{3}H/^{14}C$	^{3}H dpm	^{14}C dpm	$^{3}H/^{14}C$
UNFRACTIONATED CELLS			12.2			15.5
NON-ADHERENT CELLS (A)	113,710	11,260	10.1	164,290	9,440	17.4
ADHERENT CELLS (B) (EDTA-eluted)	26,900	400	67.3	4,650	1,820	2.6
ADHERENT CELLS (C) (retained on monolayers) [b]	14,167	227	62.7	5,069	1,105	2.6
$\frac{\text{TOTAL ADHERENT}}{\text{TOTAL RECOVERED}} \times 100$ [c]	26.5	5.3		5.6	23.7	

a Lymph node cells from HO rats immunised with AO or DA skin grafts 8 days previously were incubated on AO.TDL monolayers.

b After removal of the adherent cells eluted with EDTA the monolayers were digested *in situ* and counted for ^{3}H and ^{14}C.

c $\frac{B + C}{A+B+C} \times 100$

TABLE 5.5

THYMIDINE-LABELLED IMMUNE CELL ADHERENCE [a]

EXPT.	%HO ANTI-AO ACTIVITY ADHERENT TO AO MONOLAYERS (A) [b]	%HO ANTI-DA ACTIVITY ADHERENT TO AO MONOLAYERS (B)	SPECIFIC ADHERENCE INDEX $\frac{A}{B}$ [c]
1.	25.0	5.5	4.5
2.	20.9	5.2	4.0
3.	45.3	15.6	2.9
4.	27.1	13.8	1.96
Mean $\pm$ S.E.	29.6 $\pm$ 5.4	10.0 $\pm$ 2.7	3.3 $\pm$ 0.6

a Mixtures of HO anti-AO and HO anti-DA cells labelled with ^{3}H- and ^{14}C-thymidine as illustrated in Fig. 8 were incubated on AO.TDL monolayers.

b Per cent adherence was calculated as described in the text.

c See legend to Table 3.

the results of a representative experiment are given in detail in Table 4, and the results of four separate experiments are summarised in Table 5. Not only was there a substantial and consistent difference in the percentage of activity in the HO anti-AO and HO anti-DA cell populations which was recovered in the adherent cell fractions, but this difference was also considerably greater than was observed in the experiments using uridine-labelled cell populations.

Strictly speaking, these experiments did not measure the numbers of lymphocytes which adhered to the cell monolayers, but only the percentage of the total isotope recovered which was associated with the adherent cell fractions. There are several difficulties associated with correlating the amount of isotope present in the adherent cell fractions with the proportion of cells which adhered, the most important of which is the fact that neither radioactive uridine nor thymidine is uniformly distrubuted within labelled lymphocyte populations. Large lymphocytes incorporate relatively more uridine than do small lymphocytes, and small thymus-derived lymphocytes are in turn more heavily labelled than small thymus-independent (B) lymphocytes (Howard et al. 1972). Similarly, it is not possible to obtain homogeneous labelling of large lymphocytes with radioactive thymidine; thymidine is incorporated into DNA during the S-phase of the cell cycle and the amount and distribution of isotope within the labelled cell population is dependent on the length of time for which the cells are exposed to the isotope and the proportion of lymphocytes entering or leaving S-phase during that time. Because of the non-uniform distribution of isotope within the labelled cell populations, it was not possible to correlate the percentage of radioactivity in the adherent cell fractions directly with the numbers of lymphocytes which adhered. However, the HO anti-AO and HO anti-DA cell populations used in these experiments were obtained from the same

source viz. the lymph nodes draining the site of skin allografts and the distribution of both radioactive uridine and thymidine would be very similar in the two cell populations. The difference in the percentages of activity recovered in the adherent cell fractions can therefore be considered to reflect a difference in the proportions of cells which adhered. This being so, these experiments showed that a significantly greater proportion of HO anti-AO cells adhered to AO monolayers than did HO anti-DA cells and that the difference in the proportions of the two cell populations which adhered was significantly greater when the radioactive label was confined to those cells in the draining lymph nodes which had responded to the antigens of the skin graft.

It can be inferred that the difference in the proportion of HO anti-AO and HO anti-DA cells which adhered to AO monolayers is not likely to reflect the adherence of GvH-reactive cells of anti-AO specificity for the following reasons: in the first place, the functional studies failed to demonstrate any antigen specific adherence of GvH-reactive cells in either non-immune or immune cell populations, i.e. there was no evidence that the binding affinity of GvH-reactive cells was increased by immunisation. Secondly, the GvH activity of rat lymphocytes, as assessed by the popliteal node weight assay, is only slightly increased (by not more than 30%) as a consequence of immunisation with major histocompatibility antigens (Ford and Simonsen 1971). It is, therefore, unlikely that specific immunisation increased the frequency of GvH-reactive cells in the HO anti-AO cell populations by an amount sufficient to explain the 300% difference in the proportion of thymidine-labelled HO anti-AO and HO anti-DA cells which adhered. The difference in the proportion of the two cell populations which adhered will reflect the adherence of cytotoxic lymphocytes of monolayer

specificity, but may also reflect the specific binding of other alloantigen-reactive cells, e.g. precursors of cytotoxic lymphocytes or alloantibody-forming cells or their precursors. Any estimate of the proportions of cytotoxic lymphocytes present in the HO anti-AO cell populations derived from these figures would therefore be a maximum and would, in any case, be dependent on the relative distribution of isotope within the labelled lymphocyte (sub)populations.

These experiments showed that antigen-specific cell adherence could be detected on the basis of a difference in the proportions of lymphocytes from immunised animals which adhered to homologous and heterologous cell monolayers. The experiments presented in section 5.2 failed to demonstrate antigen specific adherence of unsensitised lymphocytes to allogeneic cell monolayers on the basis of an increase or decrease in the functional (GvH) activity of the adherent and non-adherent cell populations. This finding was confirmed in experiments in which the proportions of uridine-labelled non-immune lymphocytes which adhered to allogeneic and syngeneic cell monolayers was compared. The experimental design was similar to the previous (Fig. 8): AO and HO lymph node cells were labelled separately <u>in vitro</u> with either ^{3}H- or ^{14}C-uridine and simultaneous duplicate cell mixtures were prepared as before with exchange of the ^{3}H and ^{14}C between the two cell populations. The cell mixtures were then fractionated on TDL monolayers which were syngeneic to one of the cell populations in each mixture and allogeneic to the other. The different cell fractions were obtained as before and counted for ^{3}H and ^{14}C. The results of one experiment are presented in TABLE 6. The proportions of non-immune lymphocytes which adhered to allogeneic and syngeneic cell monolayers was the same; there was no change in the $^{3}H/^{14}C$ ratios in the adherent cell fractions of either

TABLE 5.6

MONOLAYER ADHERENCE OF URIDINE-LABELLED NON-IMMUNE CELL POPULATIONS[a]

	MIXTURE A ^{3}H-labelled AO cells ^{14}C-labelled HO cells			MIXTURE B ^{3}H-labelled HO cells ^{14}C-labelled AO cells		
	^{3}H dpm	^{14}C dpm	$^{3}H/^{14}C$	^{3}H dpm	^{14}C dpm	$^{3}H/^{14}C$
UNFRACTIONATED CELLS			4.5			5.8
NON-ADHERENT CELLS (A)	957,044	195,437	4.9	914,638	197,095	4.6
ADHERENT CELLS (B) (EDTA-eluted)	24,306	5,236	4.6	26,513	5,671	4.7
ADHERENT CELLS (C) (retained on monolayers)[b]	18,633	3,706	4.6	17,145	3,755	5.1
$\frac{\text{TOTAL ADHERENT}}{\text{TOTAL RECOVERED}} \times 100$ [c]	4.3	4.4		4.6	4.6	

a Pooled axillary, brachial and cervical HO lymph node cells incubated on AO.TDL monolayers.

b After removal of adherent cells eluted by EDTA the monolayers were digested *in situ* and counted for ^{3}H and ^{14}C.

c $\frac{B + C}{A+B+C} \times 100$

TABLE 5.7

URIDINE-LABELLED NON-IMMUNE CELL ADHERENCE [a]

EXPT.	PER CENT ADHERENT TO ALLOGENEIC MONOLAYERS [b]	PER CENT ADHERENT TO SYNGENEIC MONOLAYERS
1.	4.5	4.5
2.	8.0	7.6
3.	6.3	6.1
Mean $\pm$ S.E.	5.2 ± 1.5	5.0 ± 1.4

a Mixtures of AO and HO lymph node cells were labelled with ^{3}H- and ^{14}C-uridine and incubated on AO.TDL monolayers.

b Per cent adherence was calculated as described in the text.

mixture compared to the ratio in the unfractionated cells and the amount of ^{3}H and ^{14}C recovered in the adherent fractions of both mixtures was the same. The results of three separate experiments are summarised in TABLE 7. In no experiment was there a significant difference in the proportions of non-immune cells which adhered to allogeneic and syngeneic cell monolayers.

DISCUSSION

The functional studies presented here and in the previous chapter on the GvH and cytotoxic activity of immune cell populations separated on the basis of their adherence to allogeneic cell monolayers showed that cytotoxic lymphocytes and GvH-reactive cells could be distinguished by several criteria:

(1) Cytotoxic lymphocytes adhered specifically to allogeneic cell monolayers; they did not adhere to monolayers expressing alloantigens unrelated to the immunising antigens. In contrast, GvH-reactive cells in both immune and non-immune cell populations did not adhere specifically to allogeneic cell monolayers under the same adsorption conditions.

(2) Substantial depletion of cytotoxic lymphocytes from immune cell populations did not affect the GvH activity of the depleted cell populations.

(3) Enrichment in cytotoxic activity was not accompanied by a similar enrichment in GvH activity.

These results show that cytotoxic lymphocytes and GvH-reactive cells in immune cell populations differ with respect to target cell binding under these conditions of monolayer adsorption, and suggest that cytotoxic activity and GvH activity are functional attributes of distinct subpopulations of alloantigen-reactive T cells. While there are now several reports in the literature on the effect of

depletion of cytotoxic lymphocytes by monolayer adsorption on the GvH activity of the depleted cell populations, the effects of both depletion of and enrichment in cytotoxic lymphocytes on the GvH activity of the depleted and enriched cell populations has not been reported to date. The depletion studies presented here are in agreement with the findings of others (Clark and Kimura 1973, Mage and McHugh 1973) who also showed that depletion of mouse cytotoxic T cells was not accompanied by a reduction in the GvH activity of the depleted cell populations. While these results in themselves suggested that cytotoxic and GvH-reactive cells belonged to distinct cell populations, the present demonstration that cell populations enriched in cytotoxic lymphocytes were not enriched in GvH activity provides further evidence to substantiate this hypothesis.

Both the cytotoxic assay and the GvH assay used in these experiments allow quantitative comparisons of two or more cell populations. If cytotoxic activity and GvH activity were mutually exclusive functions of different T cell subpopulations, then one might expect substantial enrichment of one subpopulation (cytotoxic cells) to be accompanied by a reduction in the other (GvH-reactive cells) and, similarly, that depletion of cytotoxic lymphocytes would result in an enrichment in GvH-reactive cells, on a cell for cell basis. The failure to detect such a reciprocal enrichment and depletion in the experiments reported here can be satisfactorily explained if it is assumed that the number of cytotoxic cells present in immune cell populations is small compared to the number of GvH-reactive cells. If this were the case, theneven a five-fold enrichment in cytotoxic lymphocytes (the maximum observed in these experiments) may not increase the number of cytotoxic cells sufficiently to cause a detectable reduction in the (large) number of

GvH-reactive cells present in the same cell population.

The failure to demonstrate an enrichment in the GvH activity of cell populations which were enriched in cytotoxic lymphocytes makes it unlikely that these cells make a substantial contribution to the GvH response. Other groups have also failed to induce further proliferative responses, whether tested by GvH or MLC, in cells harvested from a one-way MLC at the peak of the cytotoxic response (Rouse and Wagner 1972) or in TDL activated in vivo to alloantigens (Sprent and Miller 1972) and it was suggested that cytotoxic lymphocytes were "end-cells" with a limited capacity for further proliferation in response to antigen. However, recent experiments by MacDonald et al. (1974) showed that cells generated in MLC and harvested at the peak of the cytotoxic response could not respond to specific antigen by further proliferation or increased cytotoxic activity, but that cells harvested later in the response could. These results suggested that the ability to respond was dependent on the state of differentiation of the cytotoxic lymphocytes. The cytotoxic cell populations used in the experiments reported here were obtained at the peak of the response to skin allografts, and the apparent non-responsiveness of these cells in the GvH reaction may be explained by the assumption that the majority of the cytotoxic cells were fully differentiated effector cells and, as such, were refractory to further stimulation by antigen.

An alternative explanation is that the cytotoxic lymphocytes were capable of proliferating in response to specific antigen, but were not effective in inducing lymph node enlargement. There is some evidence in the mouse that the capacity of cytotoxic cells to induce splenomegaly may not be directly related to the ability of these cells to proliferate in response to antigen; Rouse and Wagner (1972) showed that, although

cytotoxic cell populations were ineffective in inducing splenomegaly in F_1 hybrid mice, these cells were capable of further proliferation as measured by an increased ^3H-thymidine incorporation in the spleen early after injection. The popliteal lymph node weight assay is now known to depend largely on host cell proliferation and recruitment which begins some time after donor cell proliferation (Rolstad 1976) and it is, therefore, like the spleen weight assay, an indirect measure of the donor cell response. The stimulus to host cell proliferation is not known, but it may depend on factors other than the proportion of donor cells responding. It is possible, therefore, that cytotoxic cells, although capable of responding to antigen, provoke less host cell recruitment or proliferation than do non-cytotoxic GvH-reactive cells also present in immune cell populations, for reasons which are not yet understood.

While it seems relatively easy to demonstrate specific binding of cytotoxic T cells to allogeneic cell monolayers, it has proved much more difficult to demonstrate antigen specific receptors on the surface of virgin alloantigen-reactive T cells using monolayer adsorption techniques, although it has been shown that virgin T lymphocytes can be specifically depleted of antigen-reactive T cells <u>in vivo</u> (Ford and Atkins 1971, Dorsch and Roser 1974). In the adsorption studies reported here there was no evidence for antigen specific binding of non-immune GvH-reactive cells, either in experiments which compared the GvH activity of cells adherent to allogeneic and syngeneic monolayers, or in experiments in which cells non-adherent and adherent to allogeneic monolayers were tested for GvH activity against either the monolayer alloantigens or unrelated antigens. The failure to detect antigen specific cell adherence in these functional studies was confirmed in experiments in which the

proportions of labelled unsensitised lymphocytes which adhered to allogeneic and syngeneic monolayers was found to be the same. These results are in agreement with those of Clark and Kimura (1973), who also failed to detect a significant reduction in the GvH activity of rat lymph node cells non-adherent to allogeneic fibroblasts: similar results were obtained by these authors in a mouse allogeneic system.

These results are in contrast, however, to reports of specific depletion of GvH-reactive cells from non-immune mouse lymphocyte populations after incubation on monolayers of allogeneic fibroblasts (Lonai et al. 1973) and spleen cells (Bonavida and Kedar 1974, Mage and McHugh 1975). While it is possible that the discrepancy between the results obtained using fibroblast monolayers and those reported here can be attributed, at least in part, to differences in both the nature of the monolayers and the conditions of adsorption used, it is less easy to reconcile these results with those of Mage and McHugh, who used lymphocyte monolayers and very similar adsorption/centrifugation techniques. However, these authors failed to remove approximately half of the GvH activity from spleen cells obtained from non-immune animals, despite repeated adsorptions on allogeneic monolayers, and these results suggested that GvH-reactive cells were heterogeneous with respect to binding to target cell monolayers. This is compatible with the results of Ford et al. (1975), who found that only about half the donor lymphocytes which were selectively recruited to the spleen of irradiated AgB-incompatible rats underwent blast transformation, which raised the possibilitythat the non-transformed recruited cells were low affinity antigen-reactive cells. It seems, therefore, that GvH-reactive cells have a spectrum of affinities for target cell antigen and it is possible that the spleen cells used by Mage and McHugh

contained a greater proportion of high-affinity cells than the lymph node cell populations used in the experiments reported here.

The experiments of Lonai et al. and Mage and McHugh are important because they demonstrated that unsensitised alloantigen-reactive T cells express specific receptors for antigen on their surface. The question therefore arises: Why do cytotoxic lymphocytes adhere more effectively to allogeneic cell monolayers than either the non-cytotoxic GvH-reactive cells also present in immune cell populations, or GvH-reactive cells from unsensitised animals? It may be that the receptor for antigen on cytotoxic cells is of higher affinity than the receptor on GvH-reactive cells or, alternatively, it may be that cytotoxic cells develop an increased density of receptors of the same affinity during differentiation which results in an increased avidity of target cell binding. A second possibility is that both cell types recognise and bind the antigens of the monolayers equally well but the cytotoxic lymphocytes, on contacting antigen, receive a further stimulus as a consequence of which they become more firmly attached. There is some indirect evidence that more avid binding follows the encounter between cytotoxic effector cells and antigenic target cells: in contrast to the successful separation of cytotoxic cells and GvH-reactive cells reported here and elsewhere (Mage and McHugh 1973), it has proved difficult to separate cytotoxic cell precursors from GvH-reactive cells using similar adsorption techniques; depletion of GvH-reactive cells from unsensitised lymphoid cell populations is usually accompanied by a simultaneous depletion of cytotoxic cell precursors (Lonai et al. 1973, Bonavida and Kedar 1974). Since it is likely that cytotoxic effector cells possess antigen receptors of the same affinity as their precursors, these findings raise the possibility that the preferential binding of cytotoxic

lymphocytes may be associated with early events in the cytotoxic reaction and is not necessarily a consequence of differences in the affinity of the receptors for antigen on cytotoxic and GvH-reactive cells.

A third explanation for the failure of GvH-reactive cells to bind specifically to target cell monolayers, under conditions in which cytotoxic lymphocytes adhere, is that the antigens recognised by the different cells are not the same, and that the antigenic determinants recognised by GvH-reactive cells are poorly represented on the target cell monolayers. Although much less is known about the genetic loci within the major histocompatibility complex (MHC) in the rat, there is considerable evidence in both the mouse (Yunis and Amos 1971) and in man (Bach et al. 1972) that different but closely linked genetic determinants within the MHC control cytotoxic cell production and the capacity of cells to respond in MLC (or GvH). Analysis of human populations and families (Trinchieri et al.1973, Eijsvoogel et al. 1973) and mouse intra-H2 recombinants (Alter et al. 1973, Abbasi and Festenstein 1973) showed that the antigen specificities recognised by cytotoxic lymphocytes were identical or closely linked to the serologically defined (SD) major histocompatibility antigens, but were distinct from the determinants controlling MLC responsiveness (LD antigens). If a similar situation exists in the rat, then the failure of GvH-reactive cells to adhere specifically to thoracic-duct lymphocyte monolayers could be either because the appropriate antigens are not expressed or are poorly represented on TDL, or because the conditions of adsorption used were not suitable for the specific binding of GvH-reactive cells to these determinants.

Pre-labelling of rat lymphocytes *in vitro* with ^{3}H- and ^{14}C-uridine has been used to follow the *in vivo* migration of two different cell

populations injected simultaneously (Tilney and Ford 1974). This technique has been applied here to show that antigen specific adherence of immune lymphocytes can be detected by the simultaneous adsorption of different immune cell populations, labelled with ^{3}H and ^{14}C, on a cell monolayer which is homologous with the antigens used to immunise one of the cell populations and heterologous for the other. Whereas different non-immune cell populations adhered to TDL monolayers to the same extent, immune cell populations adhered to homologous monolayers to a greater extent than to heterologous monolayers. These experiments therefore demonstrated an increase in the numbers of antigen-reactive cells of a certain binding affinity as a consequence of specific immunisation. In this context, this technique could have a wider application, e.g. in clinical transplantation situations, in which an allograft rejection might be predicted on the basis of a prior increase in the extent of specific binding of host lymphocytes to cell monolayers of donor specificity. The development of such a test would, however, be dependent on establishing adsorption conditions under which those antigen-reactive cells which are relevant to rejection bind to the donor cell monolayers. This may prove difficult, since the evidence from the work presented here is that not all subpopulations of alloantigen-reactive cells bind antigen under the same conditions. If the current hypothesis is correct and cytotoxic T cells are responsible for the mediation of allograft destruction, then the successful application of such a test would also require that these cells be present in an accessible lymphocyte source, such as the peripheral blood, prior to their migration into the graft and/or to the destructive phase of the allograft reaction.

CHAPTER SIX

GENERAL DISCUSSION

Many of the specific points arising from the work presented here have already been discussed in the relevant chapters. The object of this section is to summarise the main findings and conclusions.

In the rat, cytotoxic lymphocyte precursors and initiator cells with specificity for a chosen AgB alloantigen have many similar properties. Both are present in the thoracic duct population, increase in numbers during the course of mixed lymphocyte culture and are selectively depleted by passage through F_1 hybrid recipients (Howard and Wilson 1974, Wilson et al. 1976). The work presented here shows that, following immunisation, cytotoxic lymphocytes and GvH reactive cells can be distinguished by in vitro binding to allogeneic lymphocyte monolayers. Cytotoxic lymphocytes obtained from the lymph nodes draining a skin allograft adhered to allogeneic cell monolayers which expressed the immunising antigens and were enriched in and depleted from the adherent and non-adherent cell populations respectively. Quantitation of enrichment and depletion was by titration of the different cell populations against a standard number of target cells and comparison of the numbers of cells required to give the same cytotoxic activity. No such enrichment and depletion of cytotoxic activity was found when lymphoid cell populations were adsorbed on cell monolayers which expressed alloantigens unrelated to the immunising antigens.

In contrast to the specific adsorption of cytotoxic lymphocytes, there was no evidence of antigen specific binding of GvH reactive cells to allogeneic monolayers under the same adsorption conditions. Quantitative comparison of the GvH activity of different cell populations by the popliteal lymph node weight assay showed that GvH reactive cells with specificity for the monolayer antigens were neither removed from the non-adherent cell populations nor were they present in the adherent cell populations in greater numbers than were GvH reactive cells with specificity for

unrelated alloantigens. This was true for the GvH reactive cells present in both nonimmune and immune cell populations. The failure to detect antigen specific binding of GvH reactive cells in these functional studies was confirmed in experiments using radioactively-labelled cell populations which showed that there was no apparent difference in the proportions of unsensitised lymphocytes which adhered to allogeneic and syngeneic cell monolayers.

The contrasting effects of monolayer adsorption on GvH and cytotoxic activity shows that cytotoxic lymphocytes and GvH reactive cells differ with respect to target cell binding under these conditions of adsorption and suggests that these activities are functional attributes of distinct subpopulations of alloantigen reactive cells. Although it was concluded that the majority of GvH reactive cells present in immune cell populations are not cytotoxic, the possibility that some cytotoxic cells contribute to GvH activity has not been excluded. The fact that cytotoxic lymphocytes comprise only a small proportion of the alloantigen reactive cells in the lymph nodes draining a skin allograft makes it difficult to assess their contribution to GvH activity. Nevertheless, the failure to demonstrate an enrichment and depletion in GvH activity in cell populations which were, respectively, enriched in and depleted of cytotoxic lymphocytes, makes it unlikely that these cells contribute to the GvH response. This conclusion is supported by the work of others who showed that cytotoxic lymphocyte populations produced during a systemic GvH reaction and present in mixed lymphocyte culture at the peak of cytotoxic activity were unresponsive to further stimulation with antigen, whether tested in GvH or MLC (Sprent and Miller 1972, MacDonald et al. 1974). It would appear, therefore, that the capacity of the cytotoxic T cell subpopulation to proliferate in response to antigen is lost, at least for a time, as a result of sensitisation.

Sensitisation also results in the de novo appearance of alloantigen

reactive cells which will bind to the immunising antigen under standard conditions of adsorption. Experiments in which mixtures of lymphoid cell populations, each labelled with a different radioactive isotope, were adsorbed on lymphocyte monolayers showed that, whereas there was no difference in the proportions of unsensitised cells which adhered to allogeneic and syngeneic monolayers, there was a consistent and significant difference in the proportions of lymph node cells from immunised rats which adhered to homologous and heterologous cell monolayers. Furthermore, this difference was substantially greater when the lymph node cells were labelled with a radioactive DNA precursor, suggesting that the specifically adherent cells were rapidly proliferating lymphocytes. The functional studies showed that the increase in the numbers of specifically reactive cells was not attributable to the GvH reactive cells and was at least partly, if not wholly, attributable to the cytotoxic cell subpopulation. A similar change from poorly binding precursor cells to efficiently binding effector cells as a result of sensitisation in vitro has been described in a comparative study of the effects of monolayer adsorption on cytotoxic cells and their precursors (Neefe and Sachs 1976). These findings may be relevant to recent work which has shown that, whereas allogeneic membrane fragments induce strong cytotoxic activity in secondary MLC, the same preparations do not induce either proliferative or cytotoxic responses in primary MLC (Engers et al. 1975, Wagner et al. 1976). This apparent difference in the antigenic requirements for activation of the progenitors of cytotoxic T cells in primary and secondary responses may be related to the increased avidity of antigen binding lymphocytes demonstrated here and could be the result of a progressive selection of cells with a more efficient recognition mechanism during sensitisation. Alternatively, differentiation of cytotoxic lymphocytes may involve an increase in the concentration of specific receptors for antigen on the cell surface.

It is not yet known whether the anamnestic cytotoxic response, which can be demonstrated both *in vivo* (Canty and Wunderlich 1971) and *in vitro* (Cerottini et al. 1974), is brought about by cytotoxic lymphocytes which have persisted following sensitisation, or by differentiation of cytotoxic cells from a pool of precursors which has expanded during sensitisation. The latter explanation is compatible with the accelerated proliferative response which occurs when alloimmune lymphoid cell populations are restimulated with antigen. It has been shown that the small lymphocytes present in long term mixed lymphocyte culture, which are not cytotoxic, can be induced to become cytotoxic by exposure to antigen in the presence of inhibitors of DNA synthesis (MacDonald et al. 1975, Häyry and Andersson 1975). Whether the cells which respond to antigen under these circumstances are the cytotoxic lymphocytes (or their progeny) generated during the primary response or are derived from an independent population of "memory" cells is not known. Resolution of this point must await the development of a means of identifying cytotoxic lymphocytes and their precursors other than by their eventual effector function.

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All the experiments described in this thesis were carried out by the author alone.